THE UNIVERSITY
of EDINBURGH

Defining the impact of colonisation with Shiga toxin positive *E. coli* O157 on adaptive immunity in cattle

Amy Elizabeth Beckett

MRCVS BVetMed MSc

Thesis presented for the degree of

Doctor of Philosophy

The University of Edinburgh

2018

Declaration

The research presented in this thesis is entirely my own work unless otherwise stated in the text. The material contained in this thesis has not been submitted for any other degree or professional qualification.

Amy Elizabeth Beckett Date: 27th August 2018

Contents

List of Figures:

List of Tables:

Acknowledgements

My supervisors are the people I must thank first (Tom McNeilly, David Gally and Christian Menge). All three have helped me through the journey of my PhD. Tom McNeilly has devoted hours supporting and directing me through the process without which none of this could have been achieved.

I would like to thank the Food standard agency and Food Standard Scotland for their financial support of this project. I would also like to thank everyone in and involved with the FSS "super shedding group". They have supported me through the project and allowed me to continually see how my research fits in with the rest of the STEC O157 community/work in Scotland.

Tim Connelly and Ivan Morrison gave me a great grounding and fuelled by interest in immunology research during my time in their laboratory doing my masters project. They have also kindly donated antibodies to help with this project.

Alex Corbishley was a great inspiration and help at the start of this project.

Stephen Fitzgerald and Jason Morgan, contributed a great deal towards the project and were a great support.

David Frew was a great help with many of the laboratory techniques advising, helping and teaching me. As were others in the laboratory at MRI including Yolanda Corripio- Miyari, Francesca Nunn and Mairi Mitchel.

None of the animal trials could have been performed without the assistance of the farm staff at MRI and also the skilled HSU staff.

Also I would like to thank all David Gally's group both past and present at Roslin who I have worked with including Nadejda Lupolova, Alison Tidswell, Sharif Shaaban and especially Sean McAteer who made some of the protein preparations used in this project.

Nadine Schmidt has been a great collaborator and made the process of collaborating with the group in Germany much easier, and I would like to thank all of Christian Menge's group in Germany.

I would like to thank my parents for their ongoing support. Last of all I would like to thank my Grandad (Peter Harper) for his financial support allowing me to initially do my master's degree which led onto this PhD. Unfortunately my Grandad died in the second year of my PhD but I hope he would be proud of me for fulfilling my ambitions.

Abbreviations

AB_5 A toxin with one A subunit and 5 B subunits

ANOVA Analysis of variance

APC Antigen presenting cells

ASC Antibody secreting cell

AUD Australian Dollar

BCA Bicinchoninic acid

BCR B-cell receptor

BioSS Biomathematics and Statistics, Scotland

BLV Bovine Leukaemia Virus

cDNA Complementary deoxyribonucleic acid

CFU Colony forming units

CFU/g Colony forming units per gram

CI Confidence interval

ConA Concanavelin A

CT Cefixime –tellurite

C_T Cycle threshold

DNA Deoxyribonucleic acid

DMSO Dimethyl sulfoxide

EAEC Enteroaggregative *Escherichia coli*

E. coli *Escherichia coli*

EHEC Enterohaemorrhagic *Escherichia coli*

ELISA Enzyme-linked immunosorbent assay

Elispot Enzyme-Linked ImmunoSpot

EPEC Enteropathogenic *Escherichia coli*

EU European Union

FCS Fetal calf serum

FITC fluorescein isothiocynate

FLI Friedrich-Loeffler-Institut

FMO fluorescence minus one

FSA Food Standard Agency

g acceleration due to gravity

GAM Generalised additive model

Gb_3 Globotriaosylceramide

GIT Gastrointestinal

HBSS Hanks balance salt solution

HCL Hydrochloric acid

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hi Heat inactivated

HPS Health Protection Scotland

HRP Horseradish peroxidase

HSU High Security Unit

HUS Haemolytic uremic syndrome

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IMS Immunomagnetic separation

IPRAVE Welcome Foundation International Partnership Research Award In Veterinary Epidemiology study

IQR Interquartile range

IS Insertion sequence

kDa Kilo Daltons

L Litres

LB Lysogeny Broth

LEE Locus of enterocyte effacement

LPS Lipopolysaccharide

LSA Lymphocyte stimulation assay

M Molar

MALDI Matrix-assisted laser desorption ionization

mAb Monoclonal antibody

MEM Minimum Essential Medium

MHC Major Histocompatibility complex

MRI Moredun Research Institute

nAb Neutralising antibody

NaCl Sodium Chloride

Nal Nalidixic acid

NAL-SMAC Sorbitol MacConkey agar plates containing nalidixic acid

NaOH Sodium hydroxide

NK Natural Killer

OD Optical density

OPD o-phenylenediamine dihydrochloride

OVA Ovalbumin

O157-neg Animals testing negative for STEC O157

O157-NSS Animals shedding > 0 but < 10^4 CFU/g faeces STEC O157

O157-SS Animals shedding > 10^4 CFU/g faeces STEC O157

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PT Phage Type

PVDF Polyvinylidene difluoride

RAJ Recto-anal junction

REML Restricted maximum likelihood

RFLP Restriction fragment length polymorphism

RIN RNA Integrity number

RPMI Roswell Park Memorial Institute

RNA Ribonucleic acid

Rpm Revolutions per minute

$rStx_{MUT}$ Recombinant Shiga toxin mutant

RT-qPCR Real time quantitative polymerase chain reaction

r_s Spearman Rank-order Coefficient

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM Standard error of the mean

SERAD Scottish Executive Environment and Rural Affairs Department study

SERL Scottish *E. coli* O157/VTEC Reference Laboratory

SFU Spot forming units

STEC Shiga toxigenic *Escherichia coli*

Stx Shiga toxin

T3SP Type III secreted protein

T3SS Type III secretion system

TCA Trichloroacetic acid

T_H T helper

Tir Translocator intimin receptor

TLRs Toll-like receptors

TMB 3,3',5,5'-Tetramethylbenzidine

Tris HCl Tris (hydrocymethyl) aminomethane hydrochloric acid

TSB Tryptone soya broth

UK United Kingdom

USA United States of America

USDA United States Department of Agriculture

VCA Vero cell cytotoxicity assay

VNA Verocytotoxicity neutralisation assay

v/v Volume/volume percent

Wt Wild type

w/v Weight/volume percent

ZAP Zoonotic and animal pathogens group

Lay Summary

STEC O157 is a bacteria that infects the gastro-intestinal tract of humans. Humans become infected when animal faeces contaminates food, water and the environment. Infection in humans is by the oral route, and human to human infection is also possible. Despite efforts to reduce STEC O157 transmission to humans through revisions in the food chain and improvements in critical care that reduce fatalities we are still seeing around 250 cases a year in Scotland. Often the exact source of many of these cases is unknown, however we known that the main reservoir for human infection is the intestinal tract of ruminants, especially cattle.

Unlike humans, cattle can be infected and shed high levels of STEC O157 in their faeces without showing any signs of illness. Humans generally develop bloody diarrhoea, but because the bacteria produce toxins which can affect the blood vessels in the kidneys and brain some will go on to develop kidney failure and or neurological signs. Unfortunately it is often children who go on to develop these more severe symptoms.

Some strains of the bacteria are more able to transmit from one animal to another and also shed in animal's faeces at higher levels and thus more likely to affect humans. Although STEC O157 does not cause any clinical signs of disease in cattle, it is believed that the shiga toxins produced by this bacteria are suppressing the cattle's immune system enabling the bacteria to better colonise and survive in the cattle's gastro-intestinal tract. Understanding how the cattle's immune system responds to the bacteria, hopefully will enable better interventions to be developed to help reduce the levels of bacteria in cattle faeces and thus reduce the risk of human infection in the future. This PhD examines how cattle respond to STEC O157 infections in both the field and experimental challenges.

We determined that in the field super shedding cattle (shedding high numbers of STEC O157 in their faeces) had reduced antibody responses to a STEC O157 specific antigen (Tir) compared to cattle shedding at low levels or not shedding. We determined that toxoid vaccinated calves had increased STEC O157 flagella specific antibody responses compared to calves that were not toxoid vaccinated. These results indicate that shiga toxins in the field may be causing some immune suppression in cattle. However in the experimental trials, involving orally challenging calves with STEC O157 and monitoring their immune

responses to STEC O157 both systemically and locally in the terminal rectum, which is the primary site of colonisation of the bacteria, the results did not consistently indicate significant STEC O157 specific immune suppression.

Part of this PhD also aimed to determine if immune suppression by STEC O157 in cattle was more wide spread and if any suppression of the immune system might affects the cattle's ability to respond to vaccinations routinely used in commercial farming practices. We determined that been colonised with STEC O157 could actually enhance cattle's ability to mount an immune response to a concurrent vaccine under experimental conditions. However this response was very subtle and this affect was only seen with one of the three challenge strains used in these experimental challenges.

In conclusion this study provides some further evidence of modulation of the host immune response by STEC O157, which is strain dependent, and variable. It seems unlikely from the data in this study that STEC O157 colonisation is having a major impact on the responses of cattle to other vaccines or infections in the field.

Abstract

Shiga producing *E. coli* (STEC) O157 is a zoonotic pathogen. In humans STEC O157 causes bloody diarrhoea and potentially fatal renal failure. Cattle are the major reservoir, where bacteria are limited to the intestinal tract and do not cause clinical signs of disease. Previous studies indicate that shiga toxins produced by STEC O157, suppress STEC-specific cellular immune responses *in vivo*.

This study aimed to initially examine the humoral immune response in cattle following natural challenge and the effects of a toxoid vaccination on this humoral STEC specific-immune response. We determined a statistically significant suppression in Tir specific IgA in STEC O157 positive cattle compared to O157 negative cattle but not in super shedding cattle. Following toxoid vaccination we determined a significant increase in flagellin specific IgG_1 antibody levels in toxoid vaccinated animals despite lower numbers of positive faecal samples compared to placebo vaccinated controls. These results suggest that shiga toxins produced by STEC O157 are actively suppressing the STEC specific immune response in natural colonisation. To clarify this suppression further calves were orally challenged with STEC O157 (either a PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ strain) and their STEC specific immune responses monitored. STEC specific systemic antibody responses were variable and weak in some cases. STEC specific local antibody responses were only significantly increased following challenge with the PT21/28 Stx2a+Stx2c+ challenge. Transcripts for genes associated with immune responses, and in particular B cell activation, at the terminal rectum were analysed by reverse transcriptase quantitative PCR. Suppression of IL2RA transcripts was observed in calves challenged with PT21/28 Stx2a+Stx2c+ compared to control calves but not with the other two STEC O157 strains tested.

This study also aimed to determine the effects of cattle colonisation with STEC O157 on the immune response to a non-bacterial T-cell dependent antigen, ovalbumin (OVA). Cattle were orally challenged with either a PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ strain or unchallenged. Calves were subcutaneously immunised with OVA five days post challenge, on two separate occasions with a two week interval. Lymphocytes from lymph nodes local to the immunisation site demonstrated significantly increased OVA-specific proliferation and OVA-specific activation of $CD4^+$ and $CD8^+$ cells in calves that

were challenged with the PT21/28 Stx2c+ strain (but not with the other two challenge strains), compared to unchallenged controls. These results indicate that colonisation with STEC O157 can alter local adaptive immune responses to non-bacterial antigens in a strain dependent manner, unexpectedly enhancing the immune response rather than suppressing it. Circulating T cell responses were unaffected.

In conclusion this study provides some further evidence of adaption of the host immune response by STEC O157, which is strain dependent, and variable. It seems unlikely from the data in this study that STEC O157 colonisation is having a major impact on the responses of cattle to other vaccines or infections in the field.

Chapter 1

Introduction

1.1 Shiga toxin producing *E. coli* (STEC) O157

Escherichia coli are gram negative facultative anaerobic bacilli. There are many strains identified, some of which are pathogenic and can severely affect human health. Enterohaemorrhagic *Escherichia coli* (EHEC) are zoonotic pathogens and typically causes bloody diarrhoea in humans. Shiga toxigenic *E. coli* (STEC) produce cytotoxins called Shiga toxins (Stx) and a subset of these strains are EHECs which are also proven to be pathogenic in humans. Human infections can progress on to potentially fatal haemolytic uremic syndrome (HUS) characterised by capillary damage in the kidneys as a result of systemic Stx activity[1-3]. Stx target endothelial cells in humans, leading to the capillary damage in the kidneys. Stxs can also lead to central nervous system complications of STEC O157 infections in humans. Many human cases are sporadic but occasionally large outbreaks do occur[4].

STEC O157 is the main serotype of STEC associated with serious human disease in the UK[5]. The first human infection of STEC O157 was traced back to a fast food restaurant serving hamburgers in the USA[6]. The first outbreak of STEC O157 in the UK was in 1983[7]. The treatment for HUS is supportive as there is currently no specific suitable treatment available[8]. Patients are managed, with particular attention paid to fluid and electrolyte balance, nutritional support, treatment of anaemia, and control of hypertension, seizures and azotaemia[9,10]. STEC O157 is the leading cause of acute paediatric renal failure and patients may require life-long dialysis or a kidney transplant[8]. In Scotland (1984-2012 data) almost 50 % of cases of detected STEC O157 infections are in children under 16 years of age. Rates of infection are highest in children under 5 years, at 15 cases per 100,000 population, compared with a population average of 4.5 per 100,000 population. In cases of HUS due to STEC O157, 85 % are under 16 years of age[11]. The economic costs of STEC O157 infections

are substantial; for example in the USA, the costs of healthcare, social care, and lost productivity is estimated at $600 million per year[12]. The average cost for a person infected with STEC O157 varies greatly from $26 for an individual who does not receive medical care to $6.2 million for a person who died from HUS in the USA[13]. In 2011, the estimated annual total cost of STEC infections in Australia was (AUD) $2,633,181[14].

Cattle are the major reservoir of STEC O157, and reducing shedding levels in cattle is predicted to lower the risk of STEC O157 infection in humans[5,15]. Humans can become infected not only by consumption of contaminated food but also due to ruminant faecal contamination of the environment and water[16-18]. This latter observation is consistent with data indicating that incidences of human cases are positively associated with livestock density and the ratio of cattle to humans[19,20]. STEC O157 bacteria are capable of long term survival in manure, pasture and soil, potentially making control more difficult[21]. In contrast to humans, cattle are colonised with and shed STEC O157 without showing any clinical sign of disease[22]. The receptor for Stx cannot be detected histologically on cattle endothelial cells; in humans, endothelial cells are rich in Stx receptors, and binding of Stx to endothelial cells in the capillary of the kidneys leads to the potentially fatal HUS seen as a consequence of STEC O157 infections in humans (see Table 1). Other subclinical carriers of STEC O157 including sheep[21] and goats[23] have also been implicated in human outbreaks. STEC O157 has also been detected in other domestic species including pigs, dogs, cats and chickens[24].Wildlife species including deer and rabbits, which often share common grazing ground with cattle, can also act as reservoirs of infection[25,26].

The principle site of STEC O157 colonisation in cattle is the terminal rectum[27] (Figure 1) and shedding of STEC O157 is highly heterogeneous, with a small proportion of cattle (< 10 %) excreting > 10^4 colony forming units per gram (CFU/g) faeces[28]. These so-called "super shedders" are believed to have a significant role in the transmission and persistence of STEC O157 within cattle populations[5]. Other sites of the cattle intestinal tract can become colonised but it is believed that the colonisation of the terminal rectum is required for animals to shed STEC O157 at high levels[29]. Super shedding is influenced by the bacteria strain[30], but it is also seems likely that other factors are involved such as host genotype, phenotype and / or environmental factors. The infective dose of STEC O157 cattle is unknown, but it is known that oral exposure to < 300 CFU can result in infection and that the probability of infection increases with dose[31].

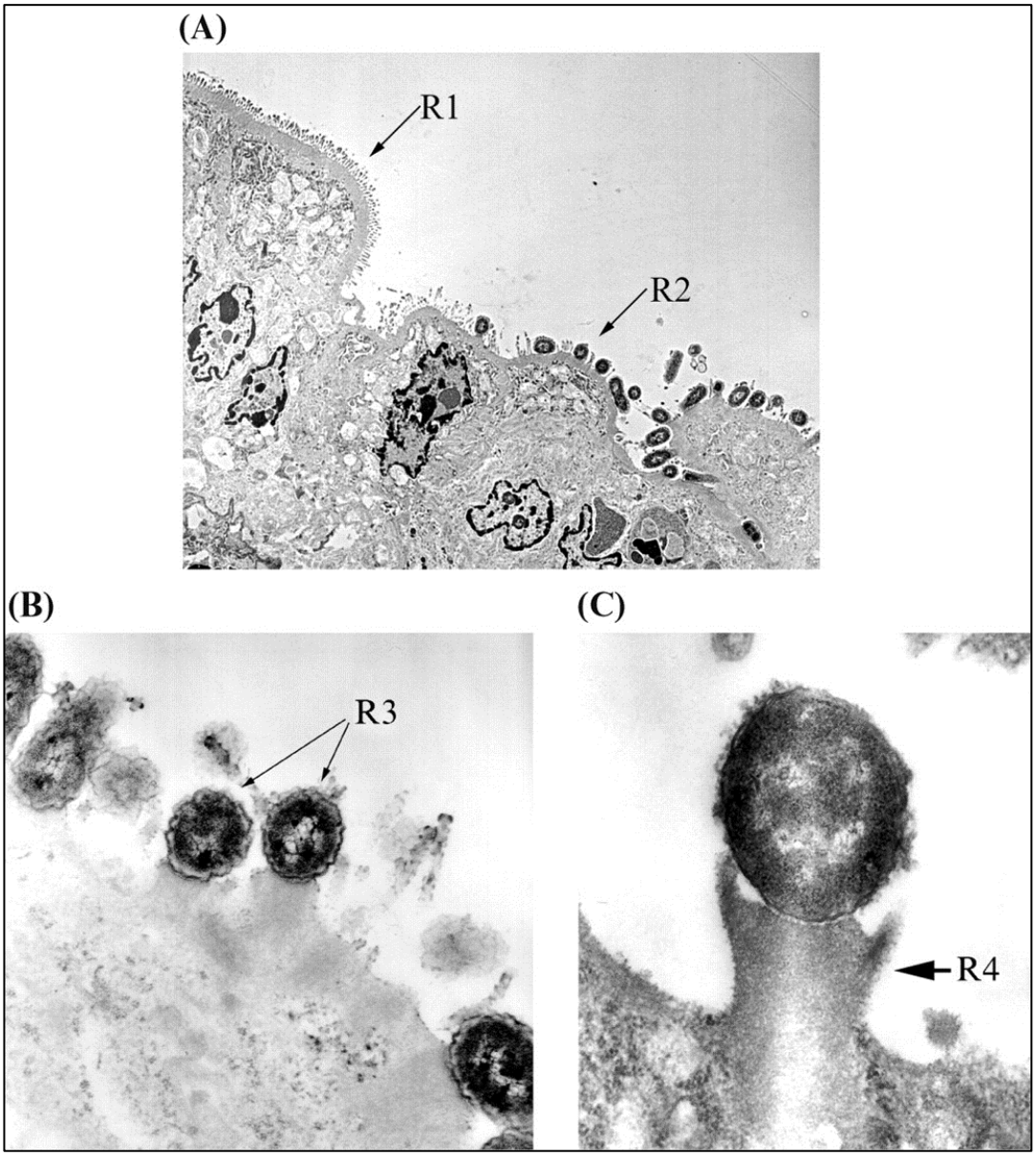


Figure 1: Transmission electron micrographs of regions containing STEC O157-positive microcolonies taken from Naylor *et al.* (2005)[32]. (A) R1 indicates a normal region of mucosa with microvilli. R2 indicates a region with attached bacteria and effaced microvilli on the terminal rectal mucosa of an experimentally colonised calf (× 1000). (B) A higher magnification than (A), showing intimately attached bacteria within an *E. coli* O157 microcolony present on the terminal rectal mucosa of an experimentally colonised calf (× 5000). (C) A pedestal formed (R4) beneath an attached bacterium within an *E. coli* O157 microcolony on the terminal rectal mucosa of a naturally colonised steer (× 10 000).

The mechanisms of pathogenicity employed by STEC O157 are numerous. The STEC O157 type III secretion system (T3SS) is one of the important virulence factors, and is involved in colonisation of the intestinal tract. It is used to deliver about 40 effector proteins such as translocated intimin receptor (Tir) into the host epithelial cells[33]. EspA forms a filamentous needle like structure and creates a physical bridge between the host cells and the bacteria. Tir is secreted from the tips of the EspA filaments[34]. Tir and intimin (a protein expressed on the bacterial cell surface) are central to the formation of intimate attaching and effacing lesions which facilitate binding of the bacteria to the cells in the gastrointestinal tract. Intimin negative strains have been shown to have an impaired ability to colonise calves which is believed to be due to their inability to form attaching and effacing lesions[35]. Other effector proteins are involved in subverting the host cell, including interfering with inflammatory signalling[36], inhibiting apoptosis and disrupting tight junctions. A pathogenicity island on the bacterial chromosome, the locus of enterocyte effacement (LEE), encodes many of these effector proteins and also the secretion system[37]. The LEE necessitates other factors encoded in a separate part of the genome to form the attaching and effacing lesions. The LEE is arranged into several polycistronic operons termed LEE1 to LEE4[38], the LEE4 operon encodes several proteins required for the attaching and effacement phenotype. Given their key role in allowing efficient colonisation of the bovine intestinal tract, T3SS proteins have been used as antigens in vaccines[39,40].

1.2 Shiga Toxins

Shiga toxins (Stx) are a family of related toxins with two major groups, Stx1 and Stx2. Stx1 and Stx2 are immunologically distinct and share only 56 % amino acid sequence identity[41]. Stx2 are more commonly associated with human disease[42] and have been found to be more potent in mice experimental challenges[43]. Stx1 is identical to the Shiga toxins of *Shigella dysenteriae* serotype 1. There are a number of variants of each Stx subtype; Stx1a, b, c and d have been identified and Stx2 a, b, c, d, e, f and g[44]. Some STEC strains produce only one type of Stx, while other strains express a combination of variants of one or both types[45]. Recent analysis of a large number of STEC O157:H7 genomes has indicated that a recent acquisition of Stx2a prophage was critical to the relatively recent emergence of STEC O157 as a life threatening zoonotic pathogen[46].

Stx consist of the enzymatic A subunit (32 kDa) and five binding B subunits (7.7 kDa)[47,48] as shown in Figure 2. The genes for the A and B subunit of Stx are carried in the late region of lysogenic bacteriophages[49]. Stx transcription starts once the bacterial SOS stress response triggers viral lytic replication of the bacteriophage. Expression during the lytic cycle removes the need for a specific secretion system since Stx is released upon phage mediated host cell lysis. Several antibiotics can lead to activation of the bacterial SOS repair response and thus the subsequent release of Stx[50,51]. This has led to the policy of not treating human cases of STEC with antibiotics due to the increased risk of Stx release leading to potentially fatal HUS[52]. The eukaryotic cell surface receptor for the B subunit of Stx is globotriasosylceramide (Gb_3/CD77)[53]. The B subunit binds to the CD77 receptor initiating endocytosis of the A subunit. The A subunit is composed of two fragments, A1 and A2, joined by a disulphide bond. A protease, furin cleaves the A subunit, releasing the A1 peptide which inactivates protein synthesis, by cleaving a *N*-glyosidic bond in the 28S subunit leading to inactivation of the 60S ribosomal unit[54].

Table 1: CD77 expression on cells in cattle and humans.

Cell	CD77 expression in human	CD77 expression in cattle
Endothelial cells	Expressed[55] (kidney, colon and brain endothelial cells)	No expression on endothelial cells *in vivo*[56]
Epithelial cells	Colonic epithelial cells low level expression[57] Expressed on mesangial and tubular epithelial cells in the kidney[55]	Expressed on some epithelial cells in the gastrointestinal tract[58] Expressed on tubular epithelial cells in the kidneys[56]
T-cells	No expression[59]	Expressed in early phase of activation[60]
B-cells	Expressed[59]	Expressed in early phase of activation[60]
Granulocytes	Expressed[61]	No expression[62]
Macrophages	Expressed[63]	Expressed[64]

In humans Stx bind to CD77 on renal glomerular endothelial, mesangial and tubular epithelial cells leading to HUS[55]. In addition to inhibiting protein synthesis, Stx are also known to induce programmed cell death, or apoptosis, in many human cell types[43]. The induction of apoptosis seems to be related to the ribotoxic stress response (due to ribosomal damage) or induction of the unfolded protein response. Stx1 shows a higher affinity for trisaccharide on CD77[65], but as previously stated Stx2 is more potent in mice and more commonly associated with disease in humans. Asymptomatic colonisation with STEC O157 in ruminants appears to be due to the lack of receptor (CD77) on endothelial cells in cattle[56] and thus Stx are unable to cause the same clinical outcome in cattle as they do in humans. Table 1 shows the known differences in Gb_3/CD77 expression on human and bovine cells.

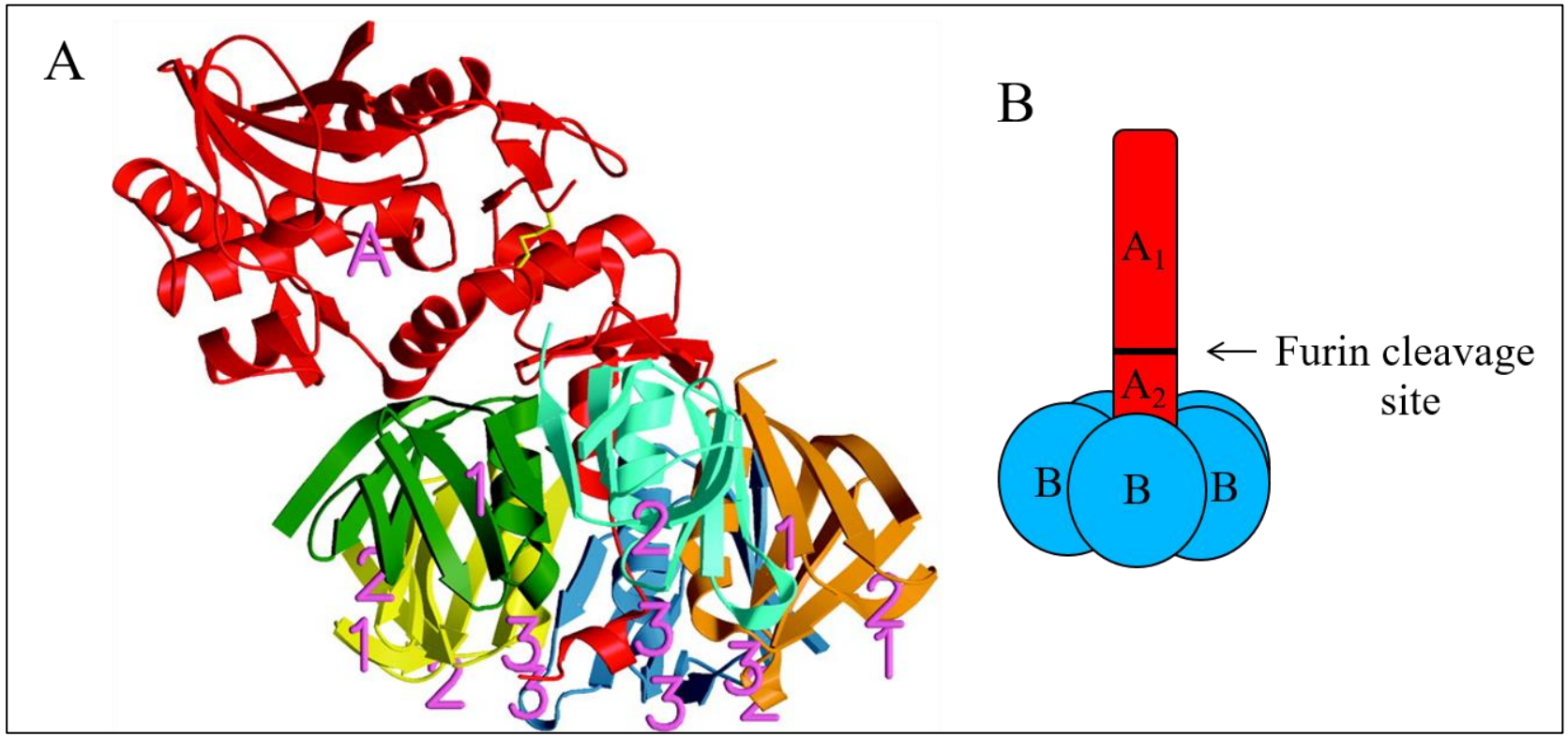


Figure 2: Diagram of Stx molecules. A is a ribbon diagram of Stx2 adapted from Fraser *et al.* (2004)[66]. The A subunit is red, whereas the B-subunits are orange, cyan, green, yellow and blue. The active site in the A-subunit is marked by the magenta letter A. The side chains of the cysteine residues that link A1 and A2 are depicted in yellow. The sites equivalent to the Gb3 binding sites on the B-pentamer of Stx1 are shown by magenta numbers that distinguish the type of binding site. B is a schematic diagram of a generic Stx molecule, the A subunit is red and the B subunits are blue.

1.3 The prevalence of STEC O157

The UK has a relatively high rate of human with STEC O157 infections[5] and Scotland is generally higher than the rest of the UK[67] as shown in Figure 3. The potential for STEC O157 to lead to large outbreaks is exacerbated by its low infectious dose in humans, believed to be less than 10 viable bacteria[68] and because some cases can be asymptomatic; however in Scotland most cases are sporadic infections. The rate of faecal culture positive cases per 100,000 population for the whole of Scotland in 2015 was 3.2, compared to 4.9 in 2014, 3.1 in 2013 and a five-year average (2010 – 2014) of 4.3[67].

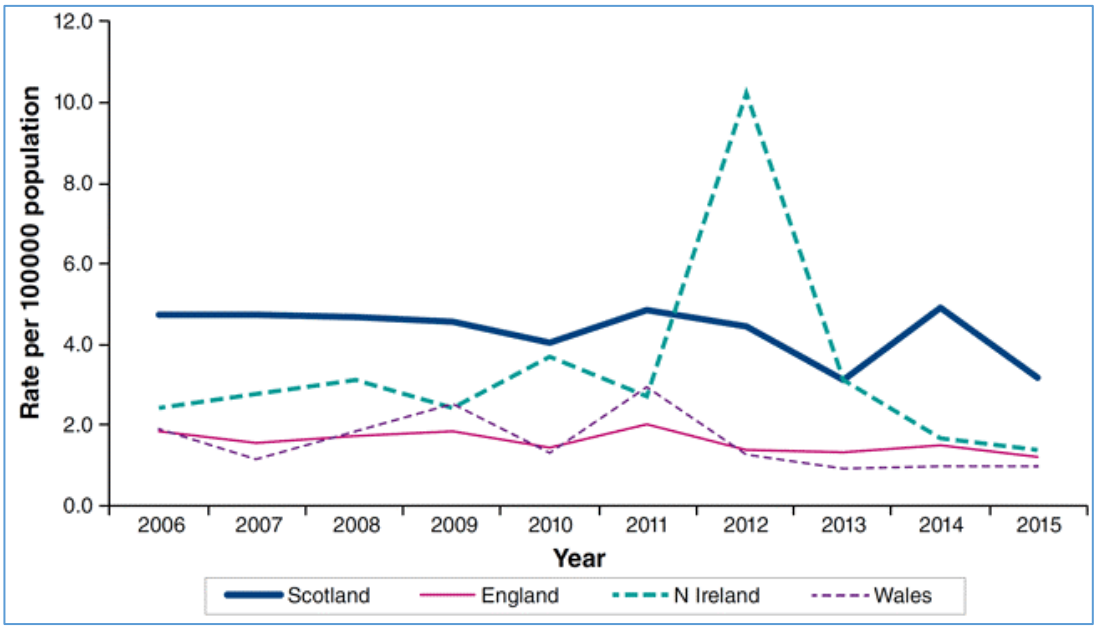


Figure 3: *E. coli* O157 rates per 100,000 population- culture positive cases, UK, 2006-2015. Adapted from Health Protection Scotland, (2016). Data for Wales, N Ireland and England includes Stx positive cases only. All data for 2015 is provisional.

Occupational, as well as recreational, exposures have been associated with infection. Health Protection Scotland (HPS) surveillance and research data show that 17 % of all *E. coli* O157 outbreaks (1996-2012), involved farms and that contact with farm animal faeces was the risk factor most strongly associated with sporadic STEC O157 outbreaks[8]. Food contamination is also a major source of infection. The surface of meat can become contaminated during slaughter and processing. Mince or ground beef products pose a particular risk if they are not

cooked properly. Unpasteurised or inadequately pasteurised milk, or raw vegetables, may also be contaminated. HPS data showed that in 28 % of all *E. coli* O157 outbreaks in Scotland (1996-2012), the main mode of transmission was foodborne[67].

The on farm prevalence of STEC O157 has been determined in two studies on Scottish farms; the Scottish Executive Environment and Rural Affairs Department study (SERAD, 1998-2000) indicated a mean farm-level prevalence of 0.218 and the Welcome Foundation International Partnership Research Award In Veterinary Epidemiology study (IPRAVE, 2002-2004) a mean farm-level prevalence of 0.205[69]. The distribution of prevalence is highly skewed, with shedding not detected in the majority of cattle groups at any time point, but a small proportion of groups contain a high number of individuals shedding bacteria in their feces[70]. A study by Omisakin *et al.* (2003) in Scotland, enumerating bacterial shedding in cattle at slaughter determined that super shedding ($\geq 10^4$ CFU/g of faeces) cattle were responsible for > 96 % of all STEC O157 bacteria shed but only made up 9 % of cattle sampled[71].

In the UK the strains of STEC O157 are subtyped by determining their sensitivity to a specific panel of 16 typing phages. In Scotland the phage type (PT) 21/28 has been most commonly associated with human disease and also more likely to be associated with super shedding in cattle[5]. PT21/28 is also a concern as it is more likely to lead to severe morbidity in human cases[72]. In Scotland between 1997 and 2001, 61 % of HUS cases in children were caused by PT21/28[72]. In one study of 88 Scottish farms in 2006, approximately half of STEC O157 isolated were PT21/28[73]. PT32 is another commonly found strain isolated in bovine faecal samples in Scotland. PT21/28 is more likely to contain both Shiga toxin 2a (Stx2a) and Shiga toxin 2c (Stx2c) encoding prophages, whereas PT32 strains mainly contain prophages encoding Stx2c alone[74]. PT32 is also less likely to be associated with high shedding levels. PT21/28 is also more likely to be identified with human disease within Scotland, suggesting that super shedding cattle strains are more likely to result in human infection[67].

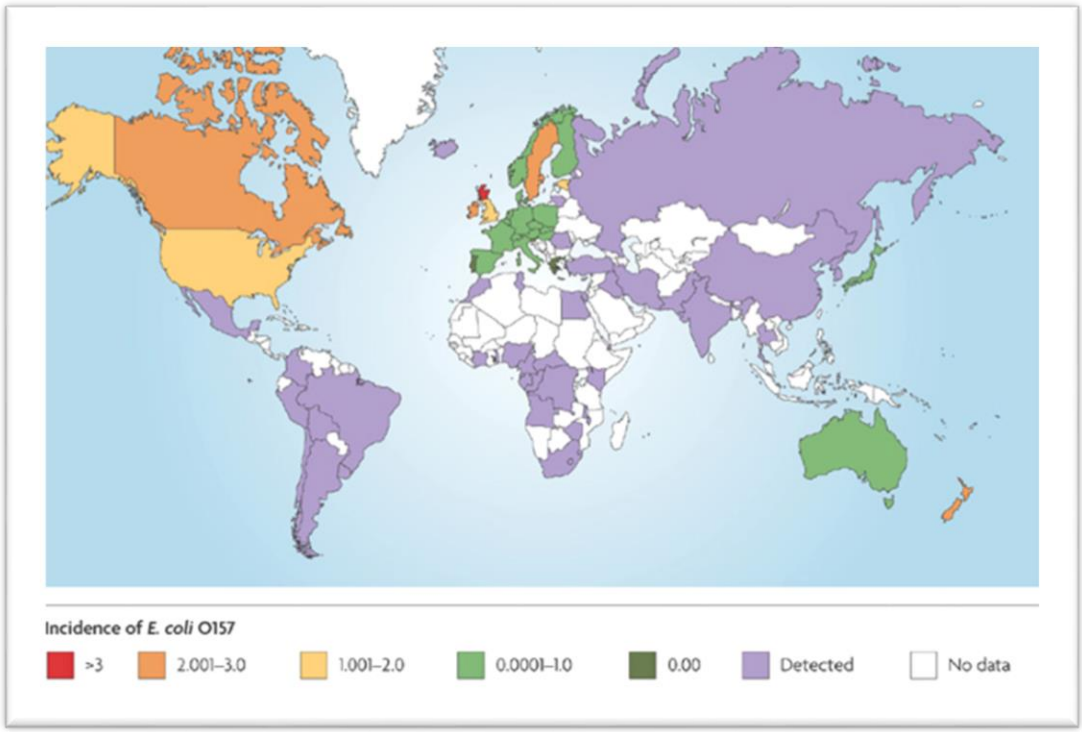


Figure 4: The worldwide human burden of STEC O157, adapted from Chase-Topping *et al.* (2008)[5]. Map of the worldwide relative burden of STEC O157 in humans in 2005 per 100,000 individuals in the population. Crude rates are presented from countries where there are surveillance programmes, although surveillance and detection methods differ between countries and therefore direct comparisons of burden are problematic.

Figure 4 demonstrates the worldwide human burden of STEC O157 in 2005. Human infections have been noted in more than 50 countries and in every continent except Antarctica[5]. The highest annual incidences are reported in the USA, Canada, Japan and Scotland[10,75]. There have been numerous studies determining prevalence of STEC O157 on farms worldwide, these studies vary in study design and laboratory methods making accurate comparisons between countries difficult. A total of 140 studies consisting of 220,427 cattle were included in a meta-analysis which estimated the prevalence of STEC O157 in individual cattle at the global level at 5.68 % (95 % confidence intervals [CI], 5.16–6.20)[76]. They also determined that world region, type of cattle, specimens and laboratory methods contributed to the variation in prevalence estimated[76]. There was a wide regional variation in the prevalence of STEC O157 in individual cattle, ranging from 1.65 % (95 % CI, 0.77–2.53) in Latin America and the Caribbean, to 31.20 % (95 % CI, 12.35–50.04) in Africa[76].

1.4 Prevalence of non-O157 serotypes

STEC O157 serotypes are the most common strains associated with Stx positive *E. coli* infections. However recent large outbreaks in the USA and Germany[77] of non-O157 strains show the potential importance of these non-O157 strains. Figure 5 demonstrates data from HPS showing a gradual increase in the number of non-O157 STEC detected in humans; and they are now identified as approximately one quarter of all laboratory confirmed infections. They have also reported that non-O157 cases can be associated with significant morbidity and include HUS cases[78]. An outbreak in Scotland in 2011, found that cases of STEC O26 were significantly more likely to develop neurological complications, diabetes mellitus and require admission to the intensive care unit than HUS cases in children due to STEC O157[78]. Although STEC O26 is still relatively uncommon in human cases in Scotland, it is a major cause of HUS in continental Europe. A Scottish study recently found a farm and pat level prevalence similar to that of STEC O157; however the prevalence of STEC O26 was reduced if the carriage of genes for virulence factors such as intimin and Stx were taken into account[79]. The outbreak of STEC O104:H4 in Germany highlights the potential for Stx prophage acquisition into different EHEC genetic backgrounds and the potential for large scale, severe outbreaks[80].

In the USA an estimated 265,000 STEC infections occur each year, O157 is the most common serotype seen in human infection (0.75 per 100,000 in 2013) but non-O157 serotypes are becoming increasingly important[81]. The big six non-O157 STEC in the USA are O26, O45, O103, O121, O111 and O145; these strains are routinely tested for in the USA[82]. In the EU in 2014, STEC was the fourth most commonly reported zoonosis, with O157 representing about half of the cases[83]. Unlike the rest of Europe, in Italy STEC O26 is the most common serotype (40 %) detected in humans, with O157 the second (33 %) and O111 (12 %) the third most common[83].

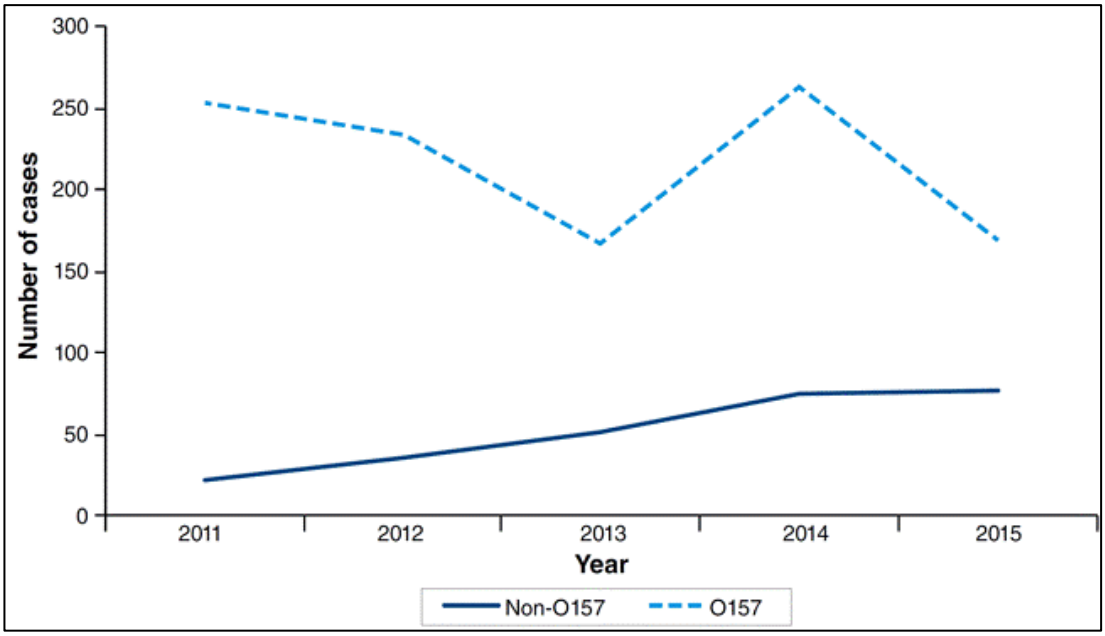


Figure 5: Faecal culture positive O157 and non-O157 isolates 2011-2015 taken from Health Protection Scotland (2016). Total number of Scottish cases in the human population.

1.5 Control of STEC O157 in cattle

Controlling STEC O157 shedding in cattle is predicted to reduce the risk of human disease[15,71]. Control methods can be broken down into pre-harvest (on farm control methods) and post- harvest (abattoir and food processing control methods).

1.5.1 Pre-harvest control of STEC O157 in cattle

The level of control of STEC O157 shedding in cattle required to protect public health is an area of debate. It has been hypothesised that controlling super shedding cattle will significantly reduce the risk to humans[15,70]. A number of control methods have been evaluated which aim to reduce STEC O157 colonisation and faecal shedding in cattle. The idea behind reducing STEC O157 in live animals (pre-harvest), is that it will reduce the burden on abattoirs and meat processing plants, thus rendering the in-plant interventions

more effective and thus reducing the risk to human health from infected meat products. The risk to humans from *Campylobacter*, another zoonotic bacterial infection has been shown to be reduced by pre-harvest on poultry farm interventions[84]. It is hypothesised that super-shedding cattle are a particular risk of allowing contamination to occur at meat processing plants[85]. Reducing STEC O157 shedding in cattle will also reduce horizontal STEC O157 spread from infected animals, reduce the STEC O157 burden in the environment and waste water streams, and it will reduce the risk to those in direct contact with animals.

A number of pre harvest interventions specifically aimed at reducing STEC O157 shedding have been considered. Antibiotics have been shown to have some level of control, and neomycin has been shown to reduce STEC O157 populations in the gastrointestinal tract[86]. However antimicrobial resistance dictates that long term use of antibiotics to control STEC O157 is an unsustainable control measure. Probiotics have also been considered as a possible control method; because competition for nutrients with endogenous or exogenous bacteria may cause inhibition of STEC O157 proliferation in the gastrointestinal tract. This is supported by evidence that faecal shedding of STEC O157 can be reduced in experimentally challenged calves when probiotic bacteria are administered[87]. In the USA a number of probiotics are routinely used including Bovamine Defend (Chr.Hansen, USA) which claim to aid in increasing production but also reducing pathogens including *E. coli* in the digestive tract[88].

Manipulation of cattle diet and feeding regimes can also reduce the carriage and shedding of STEC O157, although the effects of diet on STEC O157 shedding often appears to be inconsistent. For example some studies have found that grain fed cattle have an increased risk of shedding STEC O157[89-91] whereas another study found no significant difference between feeding a grain or forage based diet on the duration or level of STEC O157 shedding[92]. Feeding distillers grains to cattle has been shown to have a positive correlation with an increased prevalence of STEC O157[93]. Diet and probiotics could be evaluated further but changes in diet maybe unreliable and difficult to change in the modern beef industry. Probiotics although already in use have to be administered on a regular (daily) basis, which again can be difficult to deliver and even with regular delivery the effect is often variable.

Bacteriophages can be used to specifically target STEC O157 in cattle. Phages have been approved and marketed for use as a hide spray in the USA to reduce the entry of STEC O157 on the hides of cattle entering the food chain. One product Finalyse (Elanco, USA) is licenced for topical biological control of *E. coli* O157 found on the hide of cattle at harvest

facilities, it is used to spray the hides of cattle just prior to slaughter. It works in 5 minutes and its affects are reported to work for up to 4 hours. Finalyse contains a mixture of naturally occurring phages which specifically target STEC O157 and other STECs[94].

Vaccination against STEC O157 may prove to be a more reliable and deliverable control method in cattle[95]. A recent systematic review of ruminant STEC O157 vaccines concluded that vaccines targeting either epithelial adherence or iron regulation are currently the most effective[96]. Two commercial vaccines have been developed to date, both of which obtained conditional licenses for sale in North America[97]. One vaccine licensed in the USA, Epitopix SRP (Zoetis) targets siderophore proteins which the bacteria excrete in an effort to obtain iron from the intestinal tract of cattle. Targeting these proteins is thought to disrupt iron transport into the bacterium, resulting in bacterial cell death. A vaccine produced from STEC O157 extracts (type III secreted proteins, T3SPs) has been produced as Econiche (Bioniche Life Sciences Inc.) in the USA which is thought to interfere with T3SS-mediated adherence to the intestinal epithelium. However, Econiche is no longer commercially available as Bioniche Life Sciences Inc. divested its animal health division in 2014. The Econiche vaccine has been shown to have protective effects against experimental STEC O157 challenge[39,98]; however the efficacy of the vaccine in field studies has been variable[96]. A number of vaccine trials have been performed looking at the effectiveness of the Bioniche vaccine. In a small scale field trial, when three doses of vaccine were given, significant reductions in faecal shedding of STEC O157 were observed[39]. However, a subsequent large scale field trial published in 2005 failed to demonstrate any significant effect on faecal shedding of STEC O157, either at the time of the second vaccination (2 vaccines were administered 73-103 days apart) or at slaughter (2.5-5 months after the second vaccine)[99]. In a field trial using the Epitopix vaccine there was a reduction in STEC O157 shedding compared to control placebo vaccination cattle[100]. In experimental challenge trials, the Epitopix vaccine was given twice, 3 weeks apart, the calves were then orally challenged 2 weeks after the second vaccine with a 5 strain mixture of STEC O157[101]. They determined a significant reduction in the number of calves that were faecal culture positive for STEC O157 in vaccine versus control calves[101].

McNeilly *et al*. (2015) have demonstrated reduced bacterial shedding following subunit vaccination with EspA, intimin and flagellin (H7) with calves experimentally orally challenged with Stx negative *E. coli* O157[40]. This has not been demonstrated in a more natural challenge situation or with a Stx positive STEC O157 strain. Subunit vaccines are

more costly to manufacture and produce, but may lead to more consistent and better protection against STEC O157 in the future.

Toxoid vaccination is another alternative proposed mode of vaccination. A toxoid vaccination, is when the protein based toxin (in this case Stx) is rendered harmless and used as the antigen in the vaccine to elicit immunity, and has been shown to be a highly successful approach to control disease caused by other Shiga-toxigenic *E. coli.* For example, previous studies have demonstrated that vaccination of piglets with either chemically inactivated or genetically modified Stx2e is protective against Stx2e-induced oedema disease[102]. Kerner *et al.* (2015) have evaluated the biological safety *in vitro* of recombinant STEC shiga-toxoids as candidates for vaccines for use in cattle[103]. The group hypothesised that the toxoid vaccines against Stx1 and Stx2 will lead to protection against the immunosuppressive effects of the toxins, thus allowing cattle to develop a more rapid immune response to natural challenge STEC strains. Furthermore, as a shiga-toxoid based vaccine targets shared Stx across STEC strains it may be able to offer protection across different serotypes, it may be more commercially viable than a vaccine only offering protection against specific STEC serotypes. The Epitopix patent submission suggests that sequence conservation between the siderophore receptor genes provides the opportunity for vaccines manufactured from a single serotype of a bacteria to induce protective immunity against a range of serotypes and broad range of related organisms. However, these claims have not been demonstrated and importantly a field trial with Epitopix vaccine failed to demonstrate any impact of the vaccine on STEC O26 prevalence[104].

With any intervention there are potential negative consequences. One study has demonstrated a reduction in weight gain in cattle vaccinated with the Epitopix STEC O157 vaccine compared to unvaccinated control animals[105], presumably as a result of additional handling and stress on animals as a result of the vaccination regime. This is highly significant as there is little incentive for farmers to vaccinate due to the lack of clinical signs of STEC O157 colonisation in the cattle population. Any loss of productivity due to vaccination will have a further negative impact on uptake of the vaccine. Interestingly, a survey of UK farmers concluded that vaccines may be a viable option to control STEC O157 provided there was good evidence that the intervention was effective[106]. Given the issues with the lack of field efficacy of current STEC O157 vaccines, improved vaccine efficacy is required. Cattle can clear STEC O157 from the intestinal tract naturally, and therefore a greater understanding of the role of the host immune response in the clearance of the infection will allow the development of more efficacious vaccines.

1.5.2 Post- Harvest control

It can be argued that post- harvest control measures for STEC O157 may be the most logical and effective. Pre-harvest interventions are specific for particular pathogens and they all have limits in their effectiveness. Post-harvest interventions are often non-specific and may help to reduce other pathogenic bacteria as well. Cases associated with the consumption of contaminated meat, and not through environmental routes, will be most impacted by abattoir (post-harvest) interventions. Since the emergence of STEC in the USA, the beef industry has invested in STEC control in meat processing plants[107]. Although in-plant strategies have significantly reduced contamination of meat products by STEC, these processing interventions have not been perfect[108,109].

Effective prevention and control of contamination in abattoirs requires the application of good hygiene practices, the application of Hazard Analysis and Critical Control Point based management practices and risk based meat inspection practices to reduce faecal contamination of carcasses. Because none of the interventions are 100 % effective, all beef processors use a multiple hurdle intervention system of sequential interventions at different processing steps. Hides are the main source of contamination by STEC O157 of beef carcases in commercial meat processing plants[110,111]. Contamination of the carcases through faecal contamination can occur when the hides are removed and during removal of the gastro-intestinal tract. A pre-harvest treatment of the hides as discussed previously is now licenced in the USA. Washing, chemical de-hairing and treatments with antimicrobial products have all been researched and generally show some reductions in the levels of contamination[107]. Simple hygiene and good practices such as good removal of hides, keeping them separate from the carcase all help to reduce carcase contamination in the plant.

Among the numerous retail meat cuts available, ground beef possesses more risk than other intact muscle cuts, because it can also be contaminated during the grinding operation. The Food Standard Agency (FSA) in the UK provides guidelines for businesses dealing with food to help reduce the risk of cross contamination, they provide advice on keeping equipment used for raw meat and cooked meat separate, handwashing and use of disinfectants[112]. Education of the consumer in home cooking practices play a role in helping to reduce the risk of infection; again the FSA provides advice on cooking at home with a special emphasis on the cooking of burgers[113].

In practice a combination of pre and post-harvest interventions will both be important in the control of STEC O157.

1.6 Adaptive immune responses to STEC O157 in cattle

Most animals are able to clear STEC O157 infections successfully without showing clinical signs of disease. Although STEC O157 is often thought of as a normal resident of the bovine, cattle have been shown to develop an active immune response to the bacteria[97,114].

1.6.1 Systemic antibody responses

Systemic antibody responses against STEC antigens have been demonstrated to be inconsistent and are not always correlated with bacterial shedding or associated with clearance of the bacteria (Table 2). A number of studies have demonstrated systemic antibody responses to STEC antigens following oral challenge. In one study Bretschneider *et al.* (2007) demonstrated a significant increase in serum IgG specific to STEC O157 intimin, Tir, EspA, EspB and O157 LPS (lipopolysaccharide) in adult cattle following oral challenge with STEC O157 (*stx*-positive) strain. This indicates that cattle can respond serologically to STEC O157 T3SS proteins and LPS following STEC O157 colonisation. Furthermore, in this study serum IgG responses to Tir, intimin or O157 LPS were highly correlated with faecal shedding of STEC O157[115]. However a similar challenge study in calves found no correlation between anti-O157 LPS or IgG specific to Stx1 and STEC O157 shedding in oral experimentally (STEC O157, *stx*-positive) challenged calves[116,117]. The study by Bretschneider *et al*. (2007) also demonstrated a decrease in serum specific IgA to Tir, intimin and EspB following oral challenge with a STEC O157 *stx*-positive strain. The authors suggested that this may be due to sequestration of IgA from the serum by binding to the bacteria in the gastro intestinal tract of cattle. However this may also have been due to a down regulation of mucosal IgA producing cells. Antibody responses to Stx1 and Stx2 have been found in sera and colostrum in other STEC O157 oral challenge studies but the development of these antibody responses is often delayed following experimental STEC

O157 challenge[116]. Stx1 seropositivity has been demonstrated to be higher than that for Stx2[118]. It is unclear if this is due to Stx1 being expressed at higher levels than Stx2 in the gastrointestinal tract of colonised calves, or if Stx1 is more immunogenic or less immunosuppressive than Stx2. Finally, Wray *et al.* (2007) demonstrated no serological response to STEC O157 LPS following oral challenge with STEC O157 in adult cattle and variable response in calves[117].

Table 2: Studies determining antibody responses in cattle either experimentally challenged or naturally colonised with STEC O157.

Challenge	Sample	Antibody response	Correlation with bacterial shedding	Reference
Experimental STEC O157 *stx* positive oral challenge	Serum	IgG specific for intimin, Tir, EspA, EspB or O157 LPS detected	Serum IgG specific for intimin, Tir and O157 LPS positively correlated with faecal shedding	Bretschneider *et al.* (2007)
Experimental STEC O157 *stx* positive oral challenge	Serum	IgA specific for Tir, intimin and EspB decreased after oral challenge	Not calculated	Bretschneider *et al.* (2007)
Experimental STEC O157 *stx* positive oral challenge	Faecal	IgG or IgA specific for intimin, Tir, EspA, EspB or O157 LPS not detected pre or post challenge	Not calculated	Bretschneider *et al.* (2007)
Experimental STEC O157 *stx* positive oral challenge	Serum	Antibodies (IgG and IgM) specific for O157 LPS and Stx1 detected following challenge (but not Stx2)	No correlation and did not prevent re-infection	Johnson *et al.* (1996)
Experimental oral STEC O157 *stx* positive oral challenge	Serum	IgG and IgM for O157 LPS: no response in adult cattle and variable in calves (increased IgG in 3 calves and decreased IgM post challenge)	No correlation	Wray *et al.* (2000)

Table 2 continued: Studies determining antibody responses in cattle either experimentally challenged or naturally colonised with STEC O157.

Challenge	Sample	Antibody response	Correlation with bacterial shedding	Reference
Experimental STEC O157 *stx* positive oral challenge	Serum	IgG and IgA for O157 LPS and flagellin (H7) demonstrated following challenge	No correlation with bacterial shedding	Naylor *et al.* (2007)
Experimental STEC O157 (both *stx*+ and *stx*- strains) oral challenge	Serum	Antibodies to O157 LPS following challenge (no Stx2 antibodies following challenge)	Not calculated	Hoffman *et al.* (2006)
Experimental *E.coli* O157 *stx* negative oral challenge	Nasal mucosal secretions	IgA and IgG specific to H7 flagellin increase detected following challenge	Not calculated	McNeilly *et al.* (2009)
Experimental *E.coli* O157 *stx* negative oral challenge	Serum	IgA and IgG specific to H7 flagellin increase detected following challenge	Not calculated	McNeilly *et al.* (2009)
Experimental *E.coli* O157 *stx* negative oral challenge	Rectal mucosal secretions	IgA specific to H7 flagellin detected following challenge (but not IgG)	Not calculated	McNeilly *et al.* (2007)
Natural exposure	Serum	IgG and IgM specific to O157 LPS detection in STEC O157 shedding calves variable	Variable association with shedding	Cristancho *et al.* (2008)

Under conditions of natural exposure and presumptive infections STEC O157 specific antibodies are even more variable and there is often poor seroconversion. Cristancho *et al.* (2008) demonstrated variable seroconversion to O157 LPS in veal calves following shedding after natural exposure, with some calves seroconverting to O157 LPS even when no STEC O157 was isolated. This may have been due to poor detection of the bacteria or exposure of the calves to bacteria with cross reactive LPS antigenic determinants[119]. It has also been demonstrated that there is transfer of STEC specific antibodies in colostrum from dam to calves, which plays an important role in elevating serum antibodies against STEC in neonatal calves[120].

1.6.2 Mucosal antibody responses

Mucosal antibodies are believed to act as the first line of defence against many bacteria and viruses. Strong rectal mucosal IgA antibody responses have been detected to EspA, EspD, EspB, Tir, H7, OmpC and O157:H7 LPS in calves experimentally challenged with a *stx*-negative *E. coli* O157 strain[114]. Another study has examined mucosal IgA antibody responses in the rectal mucosa of calves following challenge with a *stx*-positive STEC O157 strain. The authors showed that the titres can be low and inconsistent between animals[121]. It is unclear how much protection mucosal antibodies afford in STEC O157 challenges and what the precise mechanism of this protection is. There is evidence in mice that rectal mucosa antibody levels do also reflect the responses at other sites of the intestinal tract[122123]. However in cattle and sheep there is evidence that most serum IgA is locally produced, and mucosal IgA levels are often not consistent with circulating IgA levels[124,125].

1.6.3 Cellular immune responses

Studies on the cellular immune response in cattle to STEC O157 are limited and therefore the role of cellular immunity during colonisation is largely unknown. A study demonstrated that cattle colonised with STEC O157 generate lymphoproliferative responses in peripheral blood mononuclear cells (PBMC) to heat killed Stx negative *E. coli* O157[126]. In a more recent study, oral challenge of calves with two different strains of STEC O157 (a PT32 and a PT21/28 STEC O157 strain) resulted in an increase in transcripts (IFN-γ and T-bet) associated with T helper type 1 immunity within the rectal mucosa of calves[97]. Interestingly

whilst expression of IFN-γ and T-bet peaked at 7 days post challenge in calves challenged with the PT32 strain there was a delay in the peak expression of both transcripts in the PT21/28 challenged calves, suggesting a difference in cellular immune response kinetics between the two strains. Furthermore, *ex vivo* stimulation of rectal lymph node cells from the same calves with T3SS proteins resulted in proliferation of CD4, CD8 and γδ T cells from PT21/28 challenged calves whereas with cells from the PT32 challenged calves there was an increase in proliferation of natural killer (NK), CD8 and γδ T cells[97]. These findings support the hypothesis that cattle develop a cellular response during colonisation and that this response appears to vary depending on the STEC O157 strain. A study with sheep orally inoculated with a *stx*-negative *E. coli* O157 strain, also demonstrated that the animals mount a lymphocyte proliferative response to intimin, EspA and EspB[127].

1.7 Immune modulation

STEC O157 is able to persist in the bovine intestinal system, in some cases for prolonged periods of time. It has been demonstrated that some cattle can continue to shed STEC O157 in their faeces for up to 11 months after infection[128]. Also some cattle were found to shed multiple strains of STEC O157 when studied in a longitudinal study[128]. It is hypothesised that this may be in part due to the Stx produced by STEC O157 modulating the hosts immune system and thus enabling persistent colonisation. This is consistent with the weak and variable immune responses generated following STEC O157 colonisation.

Reduced innate and adaptive immune function has also recently been demonstrated in STEC O157 super shedding cattle at the terminal rectum[129]. The study performed transcriptomic analysis of rectal tissue collected from naturally colonised adult cattle which were either super shedding STEC O157 (> 10^4 CFU/g faeces) within the previous 10 days prior to sampling or negative for STEC O157. Out of a total of 58 differentially expressed genes, most (47/58) were downregulated in super-shedding cattle, of which the majority (31/47) were associated with adaptive immune responses. The authors hypothesised that this could either be due to innate and adaptive immune responses in rectal tissue of super-shedders being inherently less effective in super-shedding cattle, thus allowing high levels of shedding to occur, or immune responses being down regulated due to factors produced by the bacteria which results in less adaptive immune cells and therefore less transcripts[129]. Another transcriptomic analysis study has recently demonstrated down regulation of immune

stimulatory factors in the ileal peyers patches and the terminal rectum from calves experimentally orally challenged[130]. This work was carried out with a *stx*-negative strain of *E. coli* O157, meaning the effects on the immune response were Stx independent. Furthermore, the *E. coli* O157 negative control animals used for comparison with the challenged calves were not the same age or weaning stage as the challenged animals, therefore the direct effect of the bacteria on the intestinal immune system is unclear.

Stxs have been hypothesised to be adapting the host immune system and there are a number of studies supporting this hypothesis. It has been demonstrated in previous studies that peripheral blood mononuclear cells (PBMC) from *E. coli* O157 *stx*-negative challenged calves developed proliferative responses to heat killed STEC O157 which also increased after re- challenge with an isogenic *stx2* positive strain[126]. The same study also showed that PBMC from STEC O157 (*stx* positive) challenged calves did not develop consistent proliferative responses to heat killed STEC O157[126]. This suggests that Stx can block induction of cellular immune responses. Since calves initially challenged with a *stx*-negative strain generated proliferative responses to heat killed STEC O157 following *stx*-positive re-challenge it was also hypothesised that the effects of Stx are limited to priming of cellular immune responses and do not block the recall of existing antigen specific cellular responses[126]. Hoffman *et al.* (2006) also hypothesised that the effect of STEC O157 infection on the bovine immune system was not a generalised one, as they demonstrated that PBMC from calves challenged with *stx*-positive or *stx*-negative isogenic strains of STEC O157 were equally capable of proliferating in response to the T-cell mitogen, Concanavalin A (ConA)[126]. The same study also found that the STEC O157 (*stx*-positive) challenge did not hinder the development of a humoral response to O157 LPS[126]. As LPS can act as T-cell independent B-cell antigen this suggests that Stx may be suppressing T-cell responses but are not able to suppress T-cell independent immune responses in the same manner.

As previously discussed following challenge with phage type PT21/28 and PT32 STEC O157 the cellular immune responses can differ[97]. In the same study cells were examined from both a lymph node (rectal) local to the site of infection and also a lymph node (pre-scapular) distant to the site of colonisation; cells from both sites differed in their response to the two STEC O157 strains. The cells from the PT32 challenged animals had reduced IFN-γ response compared to uninfected controls and also PT21/28 challenged calves, in both the local and distant lymph node cells following either mitogenic (ConA) or antigenic stimulation, suggesting that challenge with the PT32 strain can result in widespread effects on the immune system[97]. The PT21/28 strain in this study had only an active Stx2c which is

the same as the PT32 strain, so the differences seen between the two strains are not associated with differences in active Stx types. Another study looking at sheep infected with Bovine Leukaemia (BLV) virus found that oral challenge with a *stx2a*-positive STEC O157 strain resulted in reduced BLV viremia when compared to sheep challenged with an isogenic mutant Stx negative strain[131]. As BLV exhibits a major tropism for B lymphocytes both in the circulation and in lymph nodes local to the site of infection[132], this suggests that Stx2a from intestinal STEC O157 is capable of interacting with circulating immune cells as well as those in secondary lymphoid organs. These two studies suggest that in addition to modulating STEC-specific immunity, STEC O157 may have more generalised effects on ruminant immune cells throughout the body, so called bystander immune modulation. While multiple studies have evaluated the general immunomodulatory effects of helminth parasites, potent immune modulators, on their ruminant hosts[133], there is little or no information available on how STEC O157 (*stx*-positive) bacteria modulate non-STEC immunity in cattle.

The distribution of CD77, the Stx receptor on cells, as shown in Table 1 varies between human and bovine cells. In humans only a few cells express CD77 receptors; the endothelial lining of the intestine, brain, and kidney express CD77, which are the main organs targeted by Stx in humans[134]. Although human neutrophils do not express CD77, Stx can bind to surface expressed TLR4 on these cells. Unlike the CD77 receptor which binds the B subunit of Stx which results in internalisation of the toxin [3,135], TLR4 on human neutrophils interacts with A subunit of Stx and the toxin is not internalised.

Stx1 targets peripheral blood and intraepithelial lymphocytes within the gastrointestinal tract of cattle[136137]. In humans the CD77 receptor is lacking on T cells but is present on B and endothelial cells[59138]. In cattle CD77 is expressed on both B and T-cells, but it has been shown that B and T-cells only transiently express CD77 on their cell surface in an early phase of activation[60]. Granulocytes in bovines fail to express CD77 and thus seem to be resistant to the impact of Stx[62]. Stx1 has been shown to significantly reduce CD8α+ T-cell and B-cell proliferation *in vitro* in cattle[60,139]. In gastrointestinal (GIT) loop experiments it was shown that Stx1 producing STEC O157 significantly reduced the percentage of CD8α+ T-cells in the intraepithelial lymphocyte population compared to loops inoculated with a *stx1* negative mutant *E. coli* O157[140].

In addition to Stx, the bacteria also produce many other proteins that have the potential to affect the host animal's immune system. For example, STEC injects the non-LEE encoded proteins N1eB, N1eC and N1eE into the host epithelial cells via the T3SS which have been

shown in Enteropathogenic *Escherichia coli* (EPEC) strains to lead to disruption of the NF-κB pathway. The NF-κB family proteins are key regulators of inflammatory genes: following immune receptor activation the NF-κB kinase complex is stimulated, resulting in NF-κB dimers (p50 and p65) entering the nucleus and promoting the transcription of genes encoding inflammatory proteins such as IL-8, IL-1β and TNF-α[141]. N1eE inhibits IκB phosphyloration which maintains the NF-κB dimers in an inhibited state and prevents NF-κB signalling[142]. N1eB enhances the activity of N1eE[142] and N1eC a zinc protease digests p65 which also prevents NF-κB signalling[143] (Figure 6). Thus these proteins can down regulate inflammatory responses at the initial site of colonisation of the bacteria. Another effector protein EspJ produced by STEC O157 is inserted into the host epithelial cell by the T3SS and is known to inhibit opsono-phagocytosis of STEC O157 by macrophages[144].

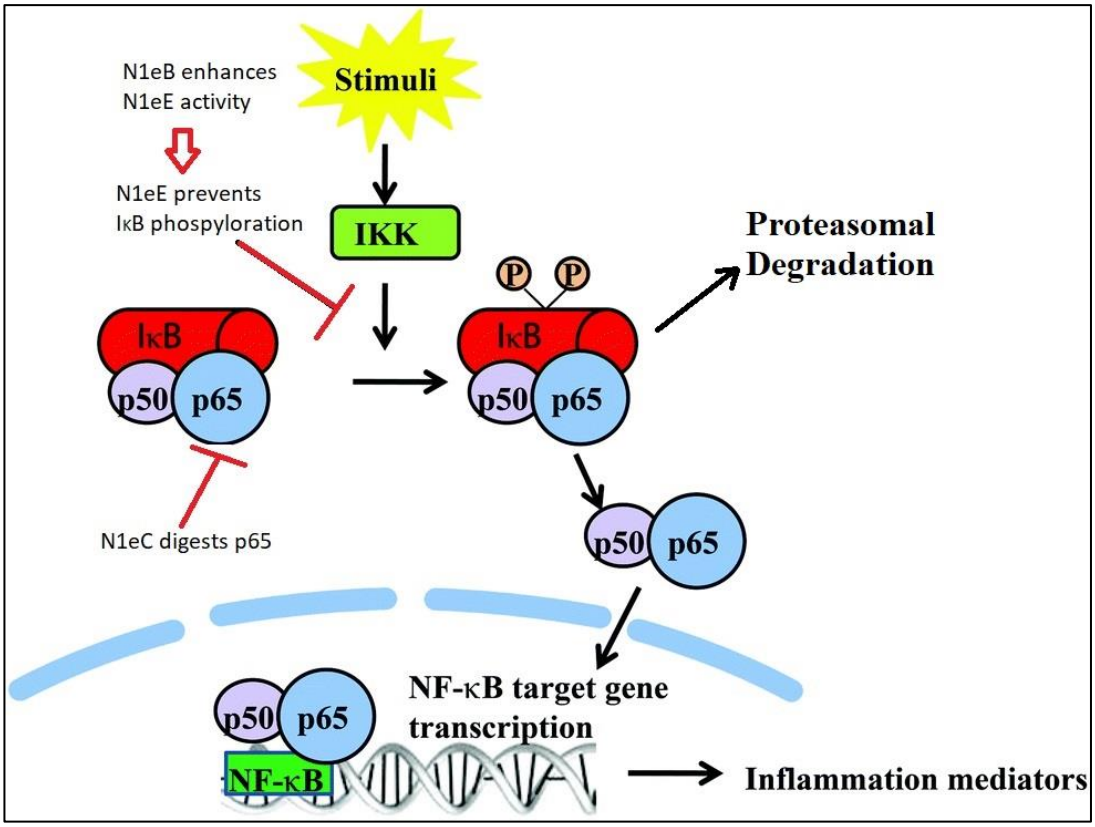


Figure 6: Schematic of NF-κB pathway adapted from Zhang *et al.* (2014)[145]. Upon stimulation, cell surface receptors activate IKK complex, which then phosphorylate IκB. These phosphorylations lead to its degradation by the proteasome and the entry of NF-κB in the nucleus, which turns on target genes. N1eE prevents the phosphorylation of IκB thus preventing NF-κB signalling, N1eB enhances the activity of N1eE. N1eC digest p65 thus also preventing NF-κB signalling.

Lymphostatin, encoded by *lifA*, is another protein which is involved in modulating host immune cells and is expressed by EPECs and many non-O157 STEC strains[146]. Lymphostatin has been shown to inhibit bovine T-cell proliferation in response to ConA[147]. STEC O157 strains lack lymphostatin, but a homologue is encoded on the pO157 plasmid, known as ToxB[148,149]. ToxB is a 365 kDa protein thought to be secreted by the T3SS, and exhibits 29.2 % identity at the amino acid level to LifA[150]. Recombinant ToxB has been shown to have lymphostatin like activity, being able to inhibit proliferation of ConA stimulated bovine T-cells[147] and IL-4 mediated activation of bovine B-cells[147]. ToxB and other effector proteins could certainly be contributing factors in immunomodulation seen by STEC O157 strains.

1.8 Conclusion

Previous studies indicate that Stx produced by STEC O157 are capable of suppressing the bovine immune system, potentially enhancing its ability to colonise the bovine intestinal tract for prolonged periods and allow super shedding in some cattle. Much of the data on antibody responses to STEC O157 infections in cattle is conflicting and studying both systemic and local antibody responses in controlled animal experiments should help to clarify this. Other pathogens that suppress host immunity to enable their longer term survival are also able to suppress host immune responses to unrelated antigens, so-called bystander immune suppression. There is a currently a lack of information in the literature about the impact of immunosuppression by STEC O157 on bystander immune responses such as those induced by concurrent infections or vaccinations.

1.9 Aims and objectives

The overall aim of this PhD is to further characterise humoral and cellular responses to STEC O157, with a focus on how *stx* positive STEC O157 modulate adaptive immune responses.

Specific aims are:

1) To characterise antibody responses to STEC O157 antigens in naturally colonised cattle including cattle that have been immunised with a Shiga-toxoid vaccine.
2) To quantify STEC-specific cellular and humoral responses following experimental challenge with STEC O157 strains expressing either Stx2a and Stx2c, or Stx2c alone.
3) To determine the effects of STEC O157 colonisation on cellular and humoral immune responses to a non-STEC O157 T-cell dependent antigen.
4) To quantify transcripts of specific genes involved in immunity at the terminal rectum of cattle during the course of STEC O157 colonisation.

Chapter 2

Natural STEC colonisation and toxoid vaccination

2.1 Introduction

In cattle, *in vitro* and *in vivo* evidence suggests that Stx play a prominent role in STEC O157 colonisation of the intestinal tract by down regulating adaptive immune responses to the bacteria[126,129,137]. Stx can suppress activation of bovine lymphocytes, in particular CD8 T-cells and B-cells, *in vitro*[126,136,137,139]. Using isogenic *stx* positive and negative strains of *E. coli* O157, Hoffman *et al.* (2006) demonstrated that challenge of calves with *stx* positive *E. coli* O157 resulted in impaired priming of circulating STEC-specific cellular immune responses, as determined by antigen-specific proliferation of peripheral blood mononuclear cells, compared to challenge with an isogenic *stx* negative *E. coli* O157 strain[126]. The duration and level of bacterial shedding was greater for calves challenged with the *stx* positive strain, demonstrating a negative association between STEC-specific cellular responses and colonisation.

The influence of Stx on humoral immunity is not as clear, Hoffman *et al.* (2006) demonstrated that challenges with *stx* positive or *stx* negative *E. coli* O157 strains induced similar levels of antibody to O157 LPS[126]. LPS is a T-cell independent antigen[151], which directly activates B-cells via engagement with Toll-like receptor 4 (TLR4) leading to proliferation and differentiation of the mature B-cells and production of antibodies. It is possible that antigens not requiring $CD4^+$ T-cell help are less sensitive to the suppressive effects of Stx. STEC protein antigens are likely to lead to T-cell dependent antibody responses; however these responses are generally weak, highly variable and often short lived, which may suggest a degree of modulation of the responses by STEC[115,121]. Bretschneider *et al.* (2007) demonstrated significant IgG responses to LEE encoding proteins

(Tir, EspA and EspB) following experimental STEC challenge but the responses to Tir and EspA were short lived[115]. The same study found a reduction in IgA serum antibody levels specific to Tir, intimin and EspB following experimental STEC challenge[115] which they speculated was a result of sequestration of STEC-specific IgA into the gut lumen and binding to STEC and its secreted proteins. Finally a recently published transcriptomic study in cattle has demonstrated that in cattle naturally colonised with STEC O157, at the terminal rectum had 31 genes associated with innate and adaptive immunity down regulated in super shedding compared to non-shedding cattle. Of these 31 down regulated genes 19 genes are directly associated with B-cell function[129]. Collectively this data suggests that STEC can actively modulate humoral immune responses. Although a role for Stx in this modulation has yet to be proven, mechanisms exist in which both T-cell dependent and independent immune responses may be modulated by Stx: either by direct interaction of Stx with B-cells[129] to modulate T-cell independent or dependent antigens, or indirectly via suppression of T-cell help[126,136] to modulate T-cell dependent responses.

Human disease can be caused by a large number of different serotypes of STEC (O157, O26, O45, O103, O121, O111 and O145) and it is believed that reducing the shedding levels of STEC in cattle by vaccination will reduce the risk of human infection[96]. To date the most promising STEC vaccines for cattle have targeted either the Type three secretion system[39,96] or bacterial iron up take mechanisms[100,101]. Evidence suggests that these vaccines may be poorly cross protective against different STEC serotypes, presumably due to serotype-dependent variation in the antigens targeted by the vaccines[152]. In order to improve cross-reactivity, a toxoid-based vaccine targeting Stx has been developed for use in cattle[103]. Kerner *et al*. (2015) generated recombinant Shiga toxoids through site-directed mutagenesis of *stx1* and *stx2a* which were devoid of immunomodulatory activity on bovine immune cells compared to recombinant wild-type toxins but retained their immunogenicity as determined by inducing antibodies following immunisation of cattle which are capable of neutralising Stx1 and Stx2 activity[103]. The group hypothesised that neutralising the immunosuppressive activities of Stx1 and Stx2 through vaccination would prevent the persistent infection with STEC in cattle[103].

This approach is predicted to cross-protect against different STEC serotypes by generating antibodies which neutralise colonisation-promoting activities of Stx such as immunomodulation[126,136,153] and enhanced epithelial colonisation[154]. By targeting the Stx with a cattle vaccination, the vaccine may also lead to protection against emerging novel

hybrid strains of STEC such as *E. coli* O104:H4 which led to nearly 900 cases of HUS and 54 deaths in 2011[155]. The *E. coli* O104:H4 strain possessed a combination of virulence factors from both STEC and enteroaggregative (EAEC) strains[155].

The hypotheses for the research presented in this chapter is that STEC colonisation modulates STEC-specific antibody responses primarily via Stx activity, and that neutralisation of Stx activity through Shiga toxoid vaccination may enhance this antibody response. To address these two hypotheses, we first determined STEC-specific antibody responses in a cohort of cattle naturally colonised with STEC O157 to determine the relationship between STEC O157 shedding and STEC-specific antibodies in the field; secondly, we determined the effects of vaccination with an experimental Stx toxoid vaccine on levels of STEC-specific antibodies in calves naturally exposed to STEC.

Aim of chapter

To characterise antibody responses to STEC O157 antigens in naturally colonised cattle including cattle that have been vaccinated with a toxoid vaccination.

2.2 Material and Methods

2.2.1 Ethics statement

The faecal sample collection during the field trial in Nebraska, USA was reviewed and approved by U.S. Meat Animal Research Centre and University of Nebraska-Lincoln Animal Care and Use Committees.

The Shiga toxoid vaccination study was carried out in strict accordance with European and German law for the care and use of experimental animals. Experiments were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany (Reg number 33.9-42502.04.12/0933).

2.2.2 Sample collection from cattle naturally colonised with STEC O157

The field samples were collected in 2011 (June to October), from an ongoing field study performed by Dr. Jim Bono (U.S. Meat Animal Research Centre, USDA, Nebraska, USA). Faecal and serum samples were collected at the same time point for each individual animal. Levels of STEC O157 shedding in the faeces were determined as described below. Shedding levels and sampling dates for individual animals are shown in Table 3. In total serum samples were available for 5 animals shedding > 10^4 CFU/g faeces STEC O157 (super shedders, O157-SS), 15 animals shedding > 0 but < 10^4 CFU/g faeces STEC O157 (positive but not super shedding, O157-NSS) and 16 STEC O157 negative animals (O157-NEG). Serum samples were stored at -20 ºC.

Table 3: STEC O157 shed in faeces from cattle in the USA field trial

Sample	Cow ID	Date	STEC O157 Status	CFU/plate	CFU/g of faeces
1	#67	11/07/2011	O157-SS	102	20400
2	#15	01/08/2011	O157-SS	130	26000
3	#41	01/08/2011	O157-SS	118	23600
4	#173	04/10/2011	O157-SS	1744	348800
5	#189	04/10/2011	O157-SS	1248	249600
6	#9	28/06/2011	O157-NSS	1	200
7	#30	28/06/2011	O157-NSS	1	200
8	#38	28/06/2011	O157-NSS	3	600
9	#78	28/06/2011	O157-NSS	13	2600
10	#80	28/06/2011	O157-NSS	3	600
11	#29	11/07/2011	O157-NSS	10	2000
12	#31	11/07/2011	O157-NSS	2	400
13	#48	11/07/2011	O157-NSS	2	400
14	#60	11/07/2011	O157-NSS	1	200
15	#65	11/07/2011	O157-NSS	2	400

Table 3 continued: STEC O157 shed in faeces from cattle in the USA field trial

Sample	Cow ID	Date	STEC O157 status	CFU/plate	CFU/g of faeces
16	#165	04/10/2011	O157-NSS	10	2000
17	#175	04/10/2011	O157-NSS	4	800
18	#178	04/10/2011	O157-NSS	11	2200
19	#187	04/10/2011	O157-NSS	5	1000
20	#214	10/04/2011	O157-NSS	28	5600
21	#2	07/11/2011	O157-NEG	0	0
22	#3	07/11/2011	O157-NEG	0	0
23	#4	07/11/2011	O157-NEG	0	0
24	#5	07/11/2011	O157-NEG	0	0
25	#6	04/10/2011	O157-NEG	0	0
26	#7	04/10/2011	O157-NEG	0	0
27	#8	04/10/2011	O157-NEG	0	0
28	#9	04/10/2011	O157-NEG	0	0
29	#10	04/10/2011	O157-NEG	0	0
30	#11	04/10/2011	O157-NEG	0	0

Table 3 continued: STEC O157 shed in faeces from cattle in the USA field trial

Sample	Cow ID	Date	STEC O157 status	CFU/plate	CFU/g of faeces
31	#1001	06/10/2011	O157-NEG	0	0
32	#1002	06/10/2011	O157-NEG	0	0
33	#1003	06/10/2011	O157-NEG	0	0
34	#1172	06/10/2011	O157-NEG	0	0
35	#1173	06/10/2011	O157-NEG	0	0
36	#1174	06/10/2011	O157-NEG	0	0

2.2.3 Sample collection from cattle immunised with recombinant Shiga toxoids

The toxoid vaccination preparation and animal trial was performed by Nadine Schmidt at the Friedrich-Loeffler-Institut, Germany, under the supervision of Prof. Dr. Christian Menge, Institute of Molecular Pathogenesis, Jena site of FLI, and of Dr. Sven Dänicke, Institute of Animal Nutrition, Brunswig, respectively. The toxoids used for vaccination were prepared as described previously[103]; briefly recombinant Stx from the *E. coli* reference strain EDL 933 (ATCC 43895) were PCR amplified, the products were ligated into a compatible plasmid vector and a QuickChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) was used to replace E167 and R170 with glutamine and leucine respectively. $rStx_{MUT}$ were expressed in *E. coli* BLR (DE3) and control preparations were obtained from *E. coli* BLR (DE3) transformed with an empty vector. After incubation of the bacterial pellet with Polymixin B, expressed toxin was collected from the periplasmic space and depleted of endotoxin (Detoxi-Gel™ Endotoxin Removing Gel, Thermo Scientific, Nidderau, Germany).

E. coli BLR (DE3) is reportedly non-motile[156] which was confirmed upon re-testing for motility using Hitchens agar. The recombinant Shiga toxoids $rStx1_{MUT}$ and $rStx2_{MUT}$ (0.75 Mio CD50-equivalent/1.4 ml/dose) was injected intramuscularly into cattle with 0.6 ml Aluminium hydroxide ($Al[OH]_3$).

The animal trial was performed as follows. Colostrum was collected from five $rStx1_{MUT}$/$rStx2_{MUT}$ vaccinated dams (at 9 and 6 weeks before their calving dates) and nine unvaccinated dams, stored (-20 °C) and pooled according to anti-Stx titres. The following year 24 calves were passively (colostrum from vaccinated cows) and actively (intra-muscularly at the 5th and 8th week of life) vaccinated. A further 24 calves served as placebo vaccinated controls (fed with low anti-Stx colostrum, placebo injected [NaCl solution and adjuvant]). During the first year of life faecal samples were collected at 3, 16, 26 and 55 weeks of age. Blood samples were collected from each animal pre and between 6 to 24 hours after initial colostrum intake, 9-12, 15-16, 15-26 and 55 weeks of age by jugular venepuncture and centrifuged; serum was subsequently collected. The serum and colostrum was stored at -20 °C and an aliquot was sent from FLI-Jena to be analysed at MRI. Figure 7 shows the time course of the vaccination and sampling schedule.

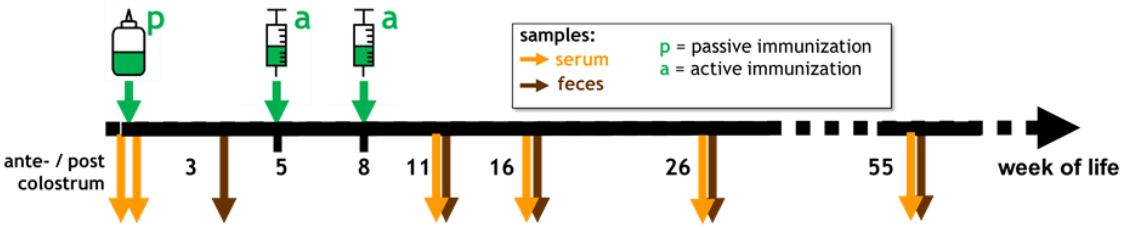


Figure 7: Time course of toxoid vaccination trial (figure from Nadine Schmidt).

2.2.4 Bacteriological analysis of STEC isolates

Bacteriology was performed at U.S. Meat Animal Research Centre, USDA, Nebraska, USA for the USDA field study, and at FLI, Jena, Germany, for the toxoid immunisation study as described below.

USDA field study:

For the faecal samples collected during the study, STEC O157:H7 was detected by immunomagnetic separation (IMS) performed according to the manufacturer's instructions using anti-EHEC O157 Dynabeads (Invitrogen, Carlsbad, USA). The number of bacteria in positive samples was enumerated by diluting faecal samples in phosphate buffered saline (PBS) and plating the dilutions onto sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC; Dalynn Biologicals, Calgary, Canada), and incubated at 37 ºC for 18 to 24 hours. Colonies were counted and expressed as CFU/g of faeces.

Shiga toxoid immunisation study:

Faecal samples were analysed for the presence of STEC by plating diluted faeces (1:10 in PBS) on Gassner agar (SIFIN diagnostics GmbH, Berlin, Germany) and incubating at 37 ºC for 18 hours. After incubation colonies were washed off with 1 ml LB broth supplemented with 30 % glycerine, boiled for 10 minutes, put on ice for 5 minutes and then used as PCR template. PCR primer pairs for detection for *stx1* and *stx2* were designed with reference to published sequence data[157]. Colonies from s*tx*-positive samples were isolated by DNA-DNA colony hybridization[158], with the following variations. DNA probes were labelled with digoxigenin with MP4/MP3 primers[157] using the PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland). The number of total *stx*-positive colonies on each blot was counted and STEC CFU/g faeces calculated.

Up to 10 *stx*-positive colonies per blot were individually cultured in 200 µl LB broth, incubated (18 hours, 37 ºC), 30 % glycerin added, and stored at -80 ºC. H-typing of 95 STEC isolates which were collected from the 3rd week (28 isolates) and 15th week (67 isolates) of the immunisation study was performed by F-fliC-1-/R-fliC2 PCR as previously described[159].

Positive PCR products were digested with RsaI (New England Biolabs GmbH, Frankfurt, Germany) and RFLP (restriction fragment length polymorphism) analysis of the resulting restriction fragment patterns was performed using Bionumerics software (Version 6.6, Applied Maths, Sint-Martens-Latem, Belgium). Isolates representing each restriction pattern and one F-fliC-1-/R-fliC2-PCR-negative isolate were sub-cultured and DNA subsequently extracted from each using a Qiagen DNeasy kit (Qiagen, Hilden, Germany) prior to microarray analysis using an *E. coli* H/O Serogenotyping AT-1 kit (Alere Technologies GmbH, Jena, Germany).

2.2.5 Vero cell cytotoxicity assay (VCA) and Verocytotoxicity neutralisation assay (VNA)

The VCA and VNA was performed by Nadine Schmidt as previously described[160] to determine the neutralising antibodies (nAb titre) against wild-type Stx1 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in serum from $rStx_{MUT}$ immunised and control calves. Briefly serum samples were tested at dilutions of 1:30 and 1:90, colostrum samples at 1:300 and 1:900. Neutralising antibody tires were determined by multiplying the relative cell activity with the dilution factor when the relative cell activity was > 30 % ($rStx1_{WT}$).

2.2.6 Generation and characterisation of STEC antigens

STEC antigens were produced as follows, the flagellins were produced by acid dissociation as previously described[161]; H21 flagellin was generated from strain ZAP0115 (O111:H21), H2 flagellin from ZAP0269 and H7 from strain ZAP984 (O157:H7). To generate H2 and H21, single colonies were used to spike motility agar and grown over night at 37 °C, the most motile colonies were picked and cultured overnight in 20 ml of LB at 37 °C (200 rpm). This was added to 1.2 L LB and cultured at 30 °C (180 rpm) until reaching optical density (OD) 600 of 1.2. The bacteria were pelleted by centrifugation at 4 °C, 10,000 *g* for 15 minutes. The pellet was re-suspended in 0.9 % NaCl with added protease inhibitor (Protease inhibitor cocktail powder, P8465, Sigma-Aldrich, Dorset, UK) overnight at 4 °C. Slowly 1 M

HCL was added with constant stirring until the pH reached 2, and stirring continued for 30 minutes. The solution was then centrifuged at 5,000 *g* for 30 minutes at room temperature. The pellet was discarded and 1 M NaOH was added to the supernatant with constant stirring until the pH reached 7. $(NH_4)_2SO_4$ was added with vigorous stirring to each 2.67 M solution, gentle stirring continued overnight at 4 ^oC. The solution was then centrifuges at 4 ^oC, 15,000 *g* for 15 minutes. The supernatant was removed and the pellet gently re-suspended in PBS and protease inhibitor (Sigma-Aldrich, Dorset, UK). The solution was dialysed overnight at 4 ^oC with PBS using cellulose tubing (Sigma-Aldrich, Dorset, UK) with a molecular weight cut off of 14,000 Daltons. H7 preparations were prepared in a similar manner by Sean McAteer (The Roslin Institute). Recombinant EspA and Tir proteins generated as previously described[40] were provided by Sean McAteer (The Roslin Institute). All proteins were analysed by One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) using a NuPAGE 4-12 % Bis-Tris Protein Gel (Invitrogen, Paisley, UK) according to the manufacturer's instructions and subsequent staining with SimplyBlue™ SafeStain (Invitrogen, Paisley, UK). The presence of LPS in each flagellin preparation was determined in a parallel gel using a Pro-Q Emerald 300 Lipopolysaccharide gel stain kit (Thermo-Fisher Scientific, Loughborough, UK) as per manufactures instructions. A trans-illuminator was used to view and image the stained gel. A bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA) was used to estimate the protein concentration in the final protein preparations.

The identities of H2 and H21 proteins were confirmed by MALDI-TOF analysis (performed by Kevin McLean, MRI) as follows: analysis was performed on protein bands of the expected molecular weight (52 and 51 kDa for H2 and H21, respectively). Selected bands were excised from the stained gels (Figure 19 A) and washed in 50mM ammonium bicarbonate in 50 % acetonitrile three times for 15 minutes each at room temperature on a vortex mixer. The solution was removed and the respective gel piece covered with 100 % acetonitrile to dehydrate for 5 minutes. Supernatant was removed and the gel piece vacuum-dried for 10 minutes. A volume of 10 mM DTT in 100 mM ammonium bicarbonate sufficient to cover the gel pieces was added and samples were placed in a 56 ^oC water bath for 1 hour. The DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and placed in the dark for 30 minutes. The iodoacetamide solution was removed and samples were given 2 ×15 minute washes with 100 µl 50 mM ammonium bicarbonate/ 50 % acetonitrile. Supernatant was removed and the gel piece vacuum-dried for 10 minutes. The gel band was then rehydrated in trypsin (Porcine

trypsin, Promega, Southampton, UK) digest solution (10 ng/µl trypsin in 25 mM ammonium bicarbonate) at 37 ºC overnight. Digests were analysed on a Bruker Ultraflex II MALDI-ToF-ToF mass spectrometer (Bruker Daltronics, Bremen, Germany), scanning the 600 to 5000 Dalton region in reflectron mode producing monoisotopic resolution. The instrument was calibrated using known peptide standards (PepMix 2, Bruker Daltronics, Bremen, Germany). Each spectrum was produced by accumulating data from 10 ×100 consecutive laser shots. Peptides were identified by matching the measured monoisotopic masses to theoretical monoisoptic masses generated using MASCOT (version 2.5.1, Matrix Science, London, UK) search engine (peptide mass fingerprinting, PMF). The search parameters were: maximum of one missed cleavage, variable modification of cysteines by propionamidation and carbamidomethylation, variable modification of methionines by oxidation, and a peptide tolerance of ± 50 ppm. Using these parameters and searching the NCBI (taxonomy *E. coli*) database, Mascot scores greater than 77 were considered significant ($p < 0.05$). See Figure 8 and 9.

Flagellin sequences were aligned using Jalview software (version 2.10.2)[162].

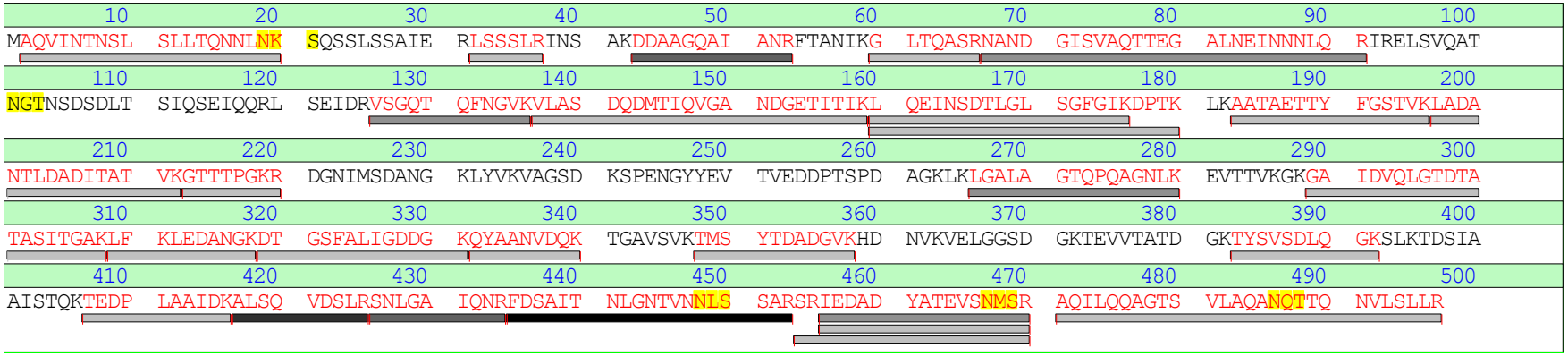


Figure 8: Results shown in ProteinScape software (Version 3.0, Bruker Daltronics, Bremen, Germany) of MALDI analysis of the excised 52 kDa band from lane 1 in figure 19 A; confirming the protein identity as H2. Amino acids in red lettering denote sequence homology between the known H2 sequence and the peptides obtained by mass spectrometry. Shades of grey boxes denote the level of intensity coverage during the mass spectrometry process from weak (pale grey) to strong (black). The sequence coverage was 68.4 %. Highlighted in yellow is the potential sites of N-glycosylation.

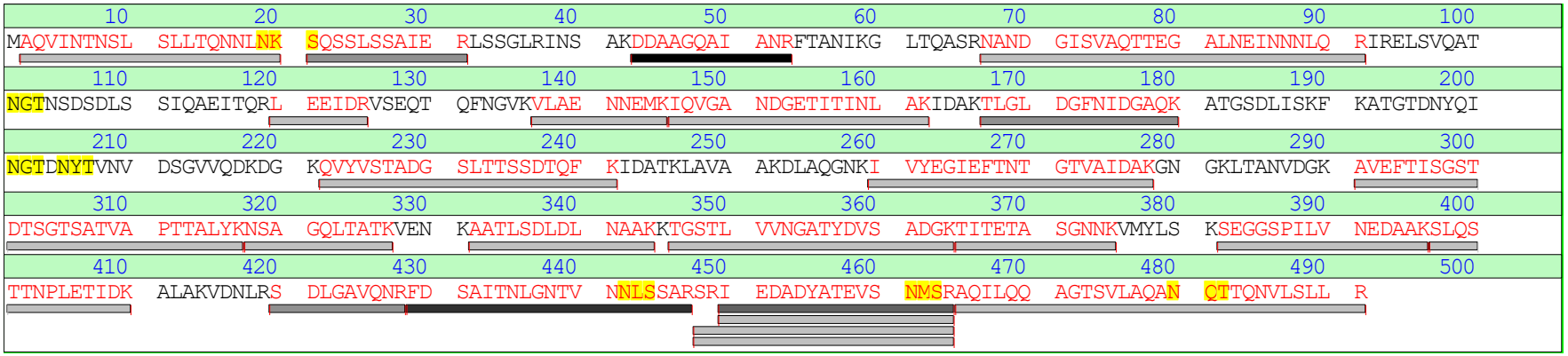


Figure 9: Results shown in ProteinScape software (version 3.0, Bruker Daltronics, Bremen, Germany) of MALDI analysis of the excised 51 kDa band from lane 3 in figure 19 A; confirming the protein identity as H21. Amino acids in red lettering denote sequence homology between the known H21 sequence and the peptides obtained by mass spectrometry. Shades of grey boxes denote the level of intensity coverage during the mass spectrometry process from weak (pale grey) to strong (black). The sequence coverage was 67.6 %. Highlighted in yellow is the potential sites of N-glycosylation.

2.2.7 Quantification of antigen-specific antibody responses

Levels of antigen-specific IgA, IgG_1 and IgG_2 in bovine serum were quantified by indirect ELISA as previously described[161] except samples were added at a set dilution (Table 4) in duplicate wells. All washes were performed using a plate washer, 5 washes with 200 µl wash buffer (PBS with 0.05 % Tween 20) per well. All incubation steps were for one hour at 37 ºC unless stated otherwise. Immulon 2HB 96-well flat bottom MicroTitre® ELISA plates (ThermoElectron Corporation, Milford, USA) were coated with antigen (H7, H2, H21, EspA or Tir) at 1 µg/ml diluted in 0.05 M carbonate buffer (1.59 g Na_2C0_3, 2.93 g NaHCO3 made up to 1 L in dH_2O, pH 9.6), 50 µl per well and incubated overnight at 4 ºC. Plates were washed and then incubated with 100 µl per well of blocking buffer (PBS with 3 % fish gelatine; Sigma-Aldrich, Dorset, UK) to prevent non-specific binding. Plates were washed again and 50 µl of diluted samples were added to each well in duplicate wells (sample dilutions, Table 2). The optimum sample dilutions were determined following serial dilution of representative samples from each group to ensure that the colour reaction at an optical density (OD) at 492 nm for the sample was on the linear part of the curve. Plates were incubated for 1 hour and then washed again; secondary antibodies (mouse anti-bovine IgA monoclonal antibody [mAb K84.2F9], anti-bovine IgG_1 [mAb K37.2G6] or anti-bovine IgG_2 [mAb K192.4F10; all AdDSerotec, Oxford, UK]) were added 50 µl per well and again the plates were incubated for 1 hour. Plates were washed again and rat anti-mouse IgG_1 conjugated to HRP (LO-MG1-2, AbDSerotec, Oxford, UK) was added 50 µl per well and the plates incubated for 1 hour. Plates were washed again and developed using o-phenylenediamine dihydrochloride (OPD [Sigma-Aldrich, Dorset, UK]) 100 µl per well and stopped after 12 minutes with stop solution (2.5 M sulphuric acid) 25 µl per well. The plates were read at 492 nm using a Tecan plate reader (Dynex Technologies, Worthing, UK). The final OD was calculated by normalising the OD to the average plate blank control OD, and inter plate variation was normalised to the OD of a positive control sample.

Colostrum samples were centrifuged at 4 ºC, 3,000 rpm for 30 minutes to separate liquid (skimmed milk) and fat. The fat layer was removed and the skimmed milk sample used for subsequent ELISAs. The dilution of colostrum samples is shown in Table 4.

Table 4: Dilution of serum samples for STEC response ELISAs

	Dilution of serum and colostrum samples		
Antigen	IgA	IgG_1	IgG_2
H7	1 in 10	1 in 100	1 in 50
Tir	1 in 10	1 in 250	1 in 25
EspA	1 in 3	1 in 25	1 in 10

2.2.8 Total Immunoglobulin levels

Total immunoglobulin levels were detected in serum samples using commercially available kits; bovine IgA, IgG_1 and IgG_2 ELISA quantitation kit (Bethyl laboratories Inc, Montgomery, USA). The manufacturer's instructions were followed except the plates were developed using 100 µl OPD for 10 minutes at room temperature and stopped with 25 µl stop solution (2.5 M sulphuric acid). OD was measured at 492 nm using a Tecan microplate reader (Dynex Technologies, Worthing, UK).

2.2.9 Western Blots

For the western blots, H2, H7 and H21 preparations (from the same batches as used in the ELISAs described above) were separated by 1D SDS-PAGE as previously described. The proteins were then transferred to a nitrocellulose membrane using an iBlot® - Western Blotting System (Invitrogen, Paisley, UK) following the manufactures instructions. The membrane was then washed three times with 20 ml of de-ionised water and then incubated

for 1 hour with 20 ml of PBS, Tween 80 (Sigma-Aldrich, Dorset, UK) and 0.5 M NaCl (PBS-T80-NaCl) with gentle rotation. All subsequent washes and dilutions were with 20 ml of PBS-T80-NaCl. The membrane was then incubated with 10 ml of pooled serum samples from animals with high (OD values of 0.8 or greater; n = 5) or low (OD values of 0.3 or lower; n = 5) levels of anti-flagellin IgG_1 (as determined by ELISA) diluted 1 in 100, for 1 hour with gentle rotation. The membranes were washed; incubated for 1 hour with 10ml mouse anti-bovine IgG_1 (diluted 1:1000 in PBS-T80-NaCl; clone K37.2G6, AbDSerotec, Oxford, UK). The membrane was washed and incubated with 10 ml of rabbit anti-mouse IgG HRP (diluted 1:1000 in PBS-T80-NaCl, P0260, Dako, Ely, UK) for 1 hour with gentle rotation. The membrane was washed again, with a final overnight wash. Bound IgG was visualised using anAmersham ECL Western Blotting Detection Kit (GE Health care life Sciences, Little Chalfont, UK) following the manufactures instructions. Images captured using an ImageQuant LAS 4000 (GE Health care life Sciences, Little Chalfont, UK).

2.2.10 Statistical analysis

A Kruskal-Wallis *H* non-parametric statistical test was used to compare antibody levels between cattle shedding different levels of STEC O157 in the USDA field study followed by Dunn's multiple comparisons test. Differences in STEC antigen specific antibody levels between toxoid vaccinated and placebo control calves at each time-point were determined by a Mann-Whitney U test. Correlations between Stx nAb titre and STEC antigen-specific antibody measures and between different H-type specific antibody levels were determined by Spearman's rank correlation test. All analyses were performed using GraphPad Prism (version 7.0, La Jolla, USA) except the Fishers exact tests which were performed on the faecal shedding data from the toxoid trial, and they were performed by Nadine Schmidt using "IBM SPSS statistics" (version 19, IBM Corporation, New York, USA).
For all statistical tests a *p*-value of < 0.05 was considered significant.

2.3 Results

2.3.1 Serum antibody responses in cattle naturally colonised with STEC O157

Serum antibody responses to selected STEC O157 antigens from cattle shedding > 10^4 CFU/g faeces STEC O157 (O157-SS), > 0 but < 10^4 CFU/g faeces STEC O157 (O157-NSS) or no detectable STEC O157 (O157-NEG) are shown in Figure 10. O157-SS cattle had significantly higher H7-specific IgA and IgG_1 compared to O157-NEG cattle. In contrast, O157-NSS cattle had lower Tir-specific IgA compared to O157-NEG cattle. No statistically significant differences in total levels of IgA, IgG_1 or IgG_2 were determined between O157-SS, O157-NSS and O157-NEG cattle (Figure 11).

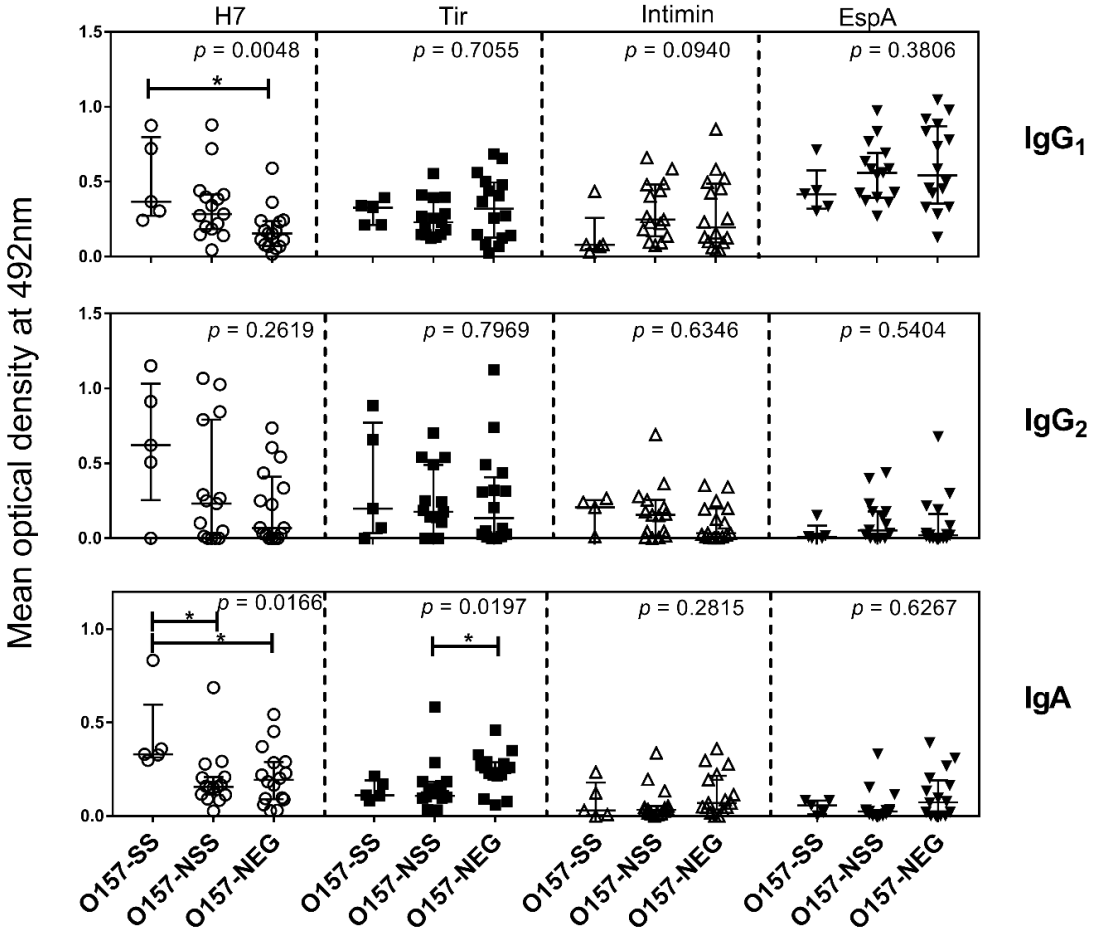


Figure 10: Antigen-specific antibody responses in cattle naturally colonised with STEC O157. Serum antibody levels were determined by ELISA. Each symbol represents the mean optical density (OD) of duplicate wells of diluted serum from one animal. The error bars represent median and interquartile range (IQR). The animals are grouped into O157-SS > 10^4 CFU/g faeces STEC O157 (n = 5), O157-NSS animals shedding > 0 but < 10^4 CFU/g faeces STEC O157 (n = 15) and STEC O157-NEG animals (n = 16). The *p*-value was determined using a Kruskal-Wallis *H* test followed by Dunn's multiple comparisons test. The overall *p*-value of the Kruskal-Wallis *H* test is shown. Statistically significant differences between the groups are also shown with * = $p < 0.05$.

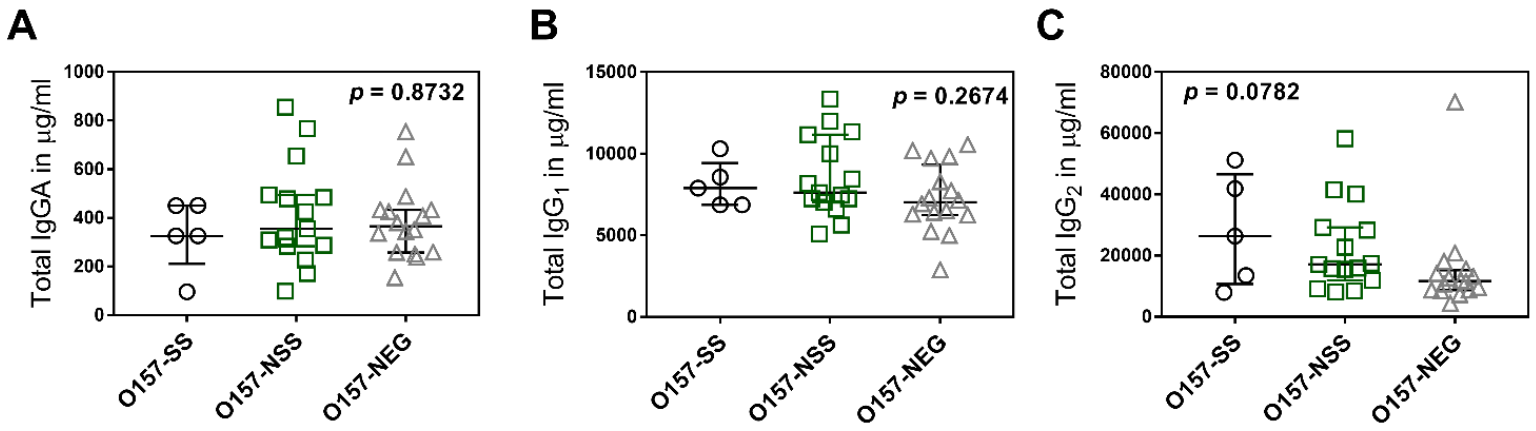


Figure 11: Total antibody levels in serum collected from cattle naturally colonised with STEC O157 determined by a commercial ELISA. Each symbol represents the mean total antibody level determined by taking the mean OD of duplicate wells of diluted serum from one animal and extrapolated the quantity from a standard curve on the same plate; (A) total IgA levels, (B) total IgG_1 and (C) total IgG_2 levels. The error bars represent median and interquartile range. The animals are grouped into O157-SS > 10^4 CFU/g faeces STEC O157 (n = 5), O157-NSS animals shedding > 0 but < 10^4 CFU/g faeces STEC O157 (n = 15) and STEC O157-NEG animals (n = 16). The overall *p*-value was determined using a Kruskal-Wallis *H* test.

2.3.2 Effects of Shiga toxoid vaccination on STEC prevalence

Figure 12 shows *stx1/stx2*-PCR results from faecal cultures for calves in the toxoid vaccination trial. *Stx1/stx2*-PCR results were positive in 34 % of faecal cultures from vaccinated calves and 49 % of faecal cultures from placebo injected control calves of all samples for each group taken over the entire observation period. A Fisher's exact test indicated a significant difference (p = 0.040) in the proportion of *stx* positive versus *stx* negative faecal samples between the toxoid vaccinated group and placebo vaccinated group over the whole observation period.

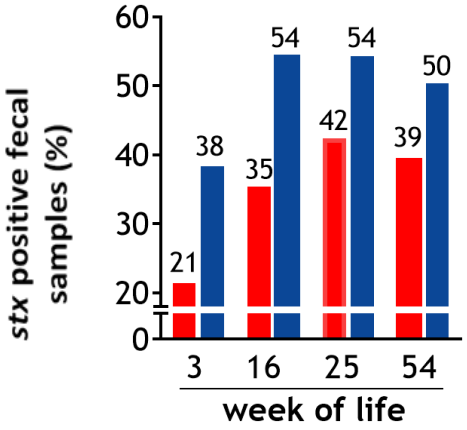


Figure 12: Assessment of the proportion of faecal samples positive for *stx1/stx2* by PCR obtained from Shiga toxoid vaccinated calves and placebo control calves. Results were positive in 34 % of overnight solid cultures of faecal samples from toxoid-vaccinated animals (red bars), 49 % control animals (blue bars) of samples (191 samples in total) taken over the entire observation period, which indicated a significantly lower proportion of *stx* positive faecal samples in the toxoid vaccinated cattle compared to the unvaccinated controls (Fisher's exact test, p = 0.040). Data, statistical analysis and figure courtesy of Nadine Schmidt, FLI, Germany.

2.3.3 Neutralising antibody levels from calves in the toxoid vaccination trial

Stx1 neutralising antibody titres for all calves in the toxoid vaccination were determined, post colostrum (24 hours), 3, 9-12, 15-16, 25-26 weeks and 1 year of age. There were statistically significant differences between the vaccinated and placebo control calves for anti-Stx1 antibodies at 24 hours, 3 weeks, 9-12 weeks and 15-16 weeks with the vaccinated group having significantly higher titres than the placebo group (Figure 13).

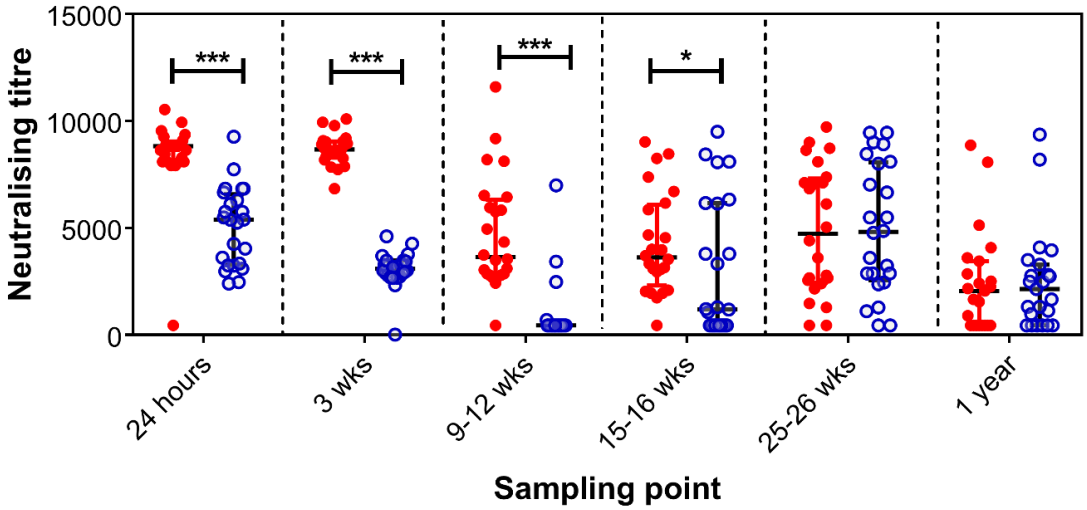


Figure 13: Temporal pattern of neutralising anti-Stx1 antibodies determined by Verocytotoxicity Neutralisation Assay (VNA). Each symbol represents an individual animal, red solid circles Stx toxoid vaccinated calves and blue clear circles placebo controls. The middle line represent median and the error bars IQR of the group. Neutralising antibody titres were compared at each time-point by Mann-Whitney U test; Significance level * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. Data courtesy of Nadine Schmidt, FLI, Germany.

2.3.4 Effects of Shiga toxoid vaccination on levels of STEC O157-antigen specific antibody responses

STEC O157-antigen specific antibody responses in the serum of vaccinated and placebo control cattle are shown in Figure 14. Following colostrum administration at 24 hours, all calves had detectable levels of H7 specific IgA. By 3 weeks the H7 specific IgA levels had rapidly decreased and remained low for the remainder of the study. The H7 specific IgA antibody levels showed statistically significant differences between the vaccinated and placebo control calves at 24 hours (p = 0.008), with the toxoid vaccinated group being significantly higher than the placebo control group. H7 specific IgA antibody responses were not significantly different between vaccinated and placebo control calves at any other time points. Again following colostrum administration at 24 hours, all calves had detectable levels of H7 specific IgG_1 with no difference between the groups. The H7 specific IgG_1 levels decreased to a low at 9-12 week, but they increased again at the later time points. There was a significant increase in H7 specific IgG_1 antibody levels in toxoid vaccinated calves compared to the placebo controls at 9-12 weeks (p = 0.0021) and 15-16 weeks (p = 0.0191). H7 specific IgG_2 antibody levels were low at the early time points and remained low until 25-26 weeks, at which stage some calves started to show increasing H7 specific IgG_2 antibody levels. There were statistically significant differences between control and toxoid vaccinated calf groups at 24 hours and 3 weeks in H7 specific IgG_2 antibody levels (p < 0.0001), with the vaccinated calves having lower levels of H7 specific IgG_2. The H7 specific IgG_2 antibody responses were no statistically significant differences between vaccinated and placebo control calves at any other time point.

Following colostrum administration at 24 hours, all calves had detectable Tir specific IgA. There were statistically significant differences in Tir specific IgA responses between vaccinated and placebo control calves at 24 hours (p < 0.0001) and also at 9-12 weeks (p < 0.05); at both time points the placebo control group was significantly higher than the toxoid vaccinated group. The Tir specific IgA responses declined after 24 hours and remained lower for the rest of the trial. The Tir specific IgA responses were not statistically significantly different between vaccinated and placebo control calves at any other time points. At 24 hours all calves had detectable Tir specific IgG_1 responses this declined until 9-12 weeks and then increased again until 25-26 weeks and then declined again by 1 year. The Tir specific IgG_1 responses were not statistically significantly different between vaccinated and placebo

control calves at any time points. Tir specific IgG_2 responses were low at 24 hours, some calves started to show responses at 15-16 weeks and the level remained similar until the end of the study. The Tir specific IgG_2 responses were not statistically significantly different between vaccinated and placebo control calves at any time points.

All calves had low EspA specific IgA responses throughout the trial. There was a statistically significant difference between vaccinated and placebo calves at 24 hours ($p = 0.0033$), being higher in the placebo control group. EspA specific IgA responses were not statistically significantly different between vaccinated and placebo calves at any other time points. All calves had detectable EspA specific IgG_1 responses at 24 hours post colostrum, the levels then decreased to a low at 9-12 weeks, increased to peak again at 25-26 weeks but had declined again by 1 year. There was a statistically significant difference in EspA specific IgG_1 responses between vaccinated and placebo control calves at 24 hours ($p = 0.023$) and 3 weeks ($p = 0.008$), with EspA-specific IgG_1 being higher in the toxoid vaccinated group. EspA specific IgG_1 responses were not statistically significantly different between vaccinated and placebo calves at any other time points. EspA specific IgG_2 responses were low until 15-16 weeks, there was no statistically significant differences between vaccinated and placebo calves at any time points.

The significant differences identified between toxoid vaccinated and placebo control calves at 24 hours generally mirrored the differences shown in the colostrum fed to the two groups, in that H7 specific IgA in the colostrum of toxoid vaccinated dams was higher than in the colostrum of control dams, H7 specific IgG_1 were similar in both colostrum groups and H7 specific IgG_2 were higher in the colostrum samples from the control dams compared to the vaccinated dams. Tir specific IgA in the control colostrum were higher than in the colostrum of vaccinated dams, Tir specific IgG_1 were very similar and Tir specific IgG_2 were higher in the control colostrum compared to the colostrum of vaccinated dams. The Tir specific antibody responses in colostrum were again mirrored in the early time points serum samples, except the IgG_2 responses which were very low in the early time point serum samples and showed no difference between the toxoid vaccinated and placebo control groups. EspA specific IgA in colostrum were higher in the control colostrum compared to that of vaccinated dams, EspA specific IgG_1 in colostrum were higher in the toxoid vaccinated group compared to controls and EspA specific IgG_2 were similar in the toxoid vaccinated group and the controls. Again these EspA specific antibody levels in colostrum correlated with differences between the toxoid vaccinated group and placebo controls in the early time

point serum samples. However, no statistical analysis was performed on the colostrum data given the low number of samples (n = 2) in each group.

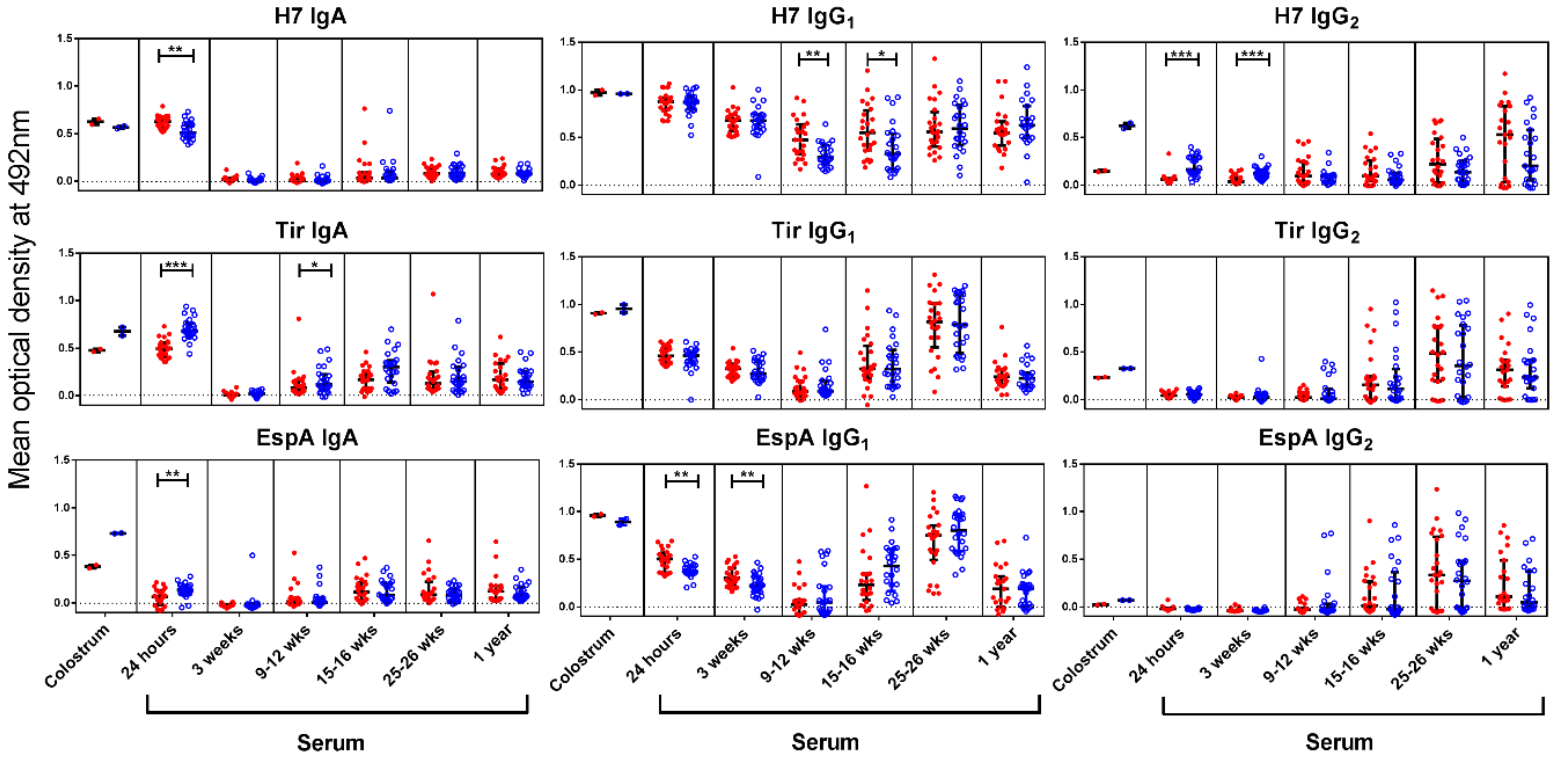


Figure 14: Colostrum and serum levels of H7, Tir and EspA antibodies in toxoid vaccinated and placebo vaccinated control calves. Each symbol represents the mean OD of duplicate wells of diluted serum from one animal. Designation "colostrum" below x-axis indicates values obtained for the colostrum pools (2 samples from each pool) used to feed the new-born calves of the respective group, time indicates calf age. The red solid symbols are the Stx toxoid vaccinated calves and the blue clear circles the control placebo vaccinated calves. The middle line represents the median and the line above and below the interquartile range. Statistical significance was determined using a Mann-Whitney U test. * p <0.05; ** p <0.01, ***p <0.001.

2.3.5 H-typing of STEC isolates from Shiga toxoid vaccinated and non-vaccinated calves

As shown in the previous section, statistically significant differences in H7 specific IgG_1 antibody were seen in the toxoid vaccinated verse control calves at 9-15 weeks, with the toxoid vaccinated calves having significantly higher H7 specific IgG_1. However, as IgG_1 responses to H7 are known to target N- and C-terminal domains which are highly conserved between different *E. coli* flagellins[161], differences in H7 specific IgG_1 between vaccinated and control calves may have reflected a general difference in antibody response to many different H-types of STEC. To determine the range of H-types of STEC that the calves may have been exposed to, H-typing was performed on a subset of STEC isolates by RFLP analysis of PCR products amplified using pan-fliC primers (F-fliC-1-/R-fliC2-PCR) and subsequent microarray analysis. Of 95 isolates collected during the vaccine trial, 3 were unavailable, so 92 were analysed; 58 isolates were positive and 34 negative by F-fliC-1-/R-fliC2-PCR. From the positive PCR products 17 different *Rsa*I- restriction digest patterns were identified. A selection of 16 "pattern" representatives and 8 "negative" representatives were used for microarray analysis. The results in Table 5 indicated that the pre-dominant H-types detected at 3 weeks were H9 and H18. At 15 weeks (closest to the time points when the differences in H7-specific IgG_1 levels were detected) the pre-dominant H-types were H2, H7 and H21.

Table 5: H-typing of STEC isolated from Shiga toxoid immunisation study by RFLP and microarray analysis

Week of life	Vaccination	Animal number	Number of isolates	Detected H antigen types
3	$rStx_{MUT}$	808	10	H1, H4, H18
		844	2	H9,
		268	2	H18, H19
3	Placebo	821	10	H18,
		819	1	H21
		826	2	H11
		273	1	H9
15	$rStx_{MUT}$	838	2	H2/H19*
		842	10	H7
		806	10	H21, H28, H33, H2/H19* H1/H12*
		811	7	H19, H21
15	Placebo	843	12	H7, H2/H19*
		267	3	H7
		819	9	H2, H49, H1/H12*
		837	2	H21
		845	10	H2

* = H-Type unclear

2.3.6 Effects of Shiga toxoid vaccination on levels of STEC-O157 antigen specific antibody responses

As the predominant H types in the study population were determined as H7, H2 and H21, antibody levels specific to H2 and H21 were also quantified to determine if the differences (between vaccinated and placebo control calves) seen in H7 specific IgG_1 antibody levels between 9-16 weeks were specific for H7 or were similar for other STEC flagellins.

H2 and H21 specific IgG_1 levels were significantly higher in the toxoid vaccinated calves at week 9-12 compared to the placebo injected controls ($p < 0.0001$ for both antigens). There were also significantly higher levels of H21 specific IgG_1 at week 15-16 ($p < 0.0001$). These results largely mirrored those seen with the H7 specific IgG_1 (Figure 15).

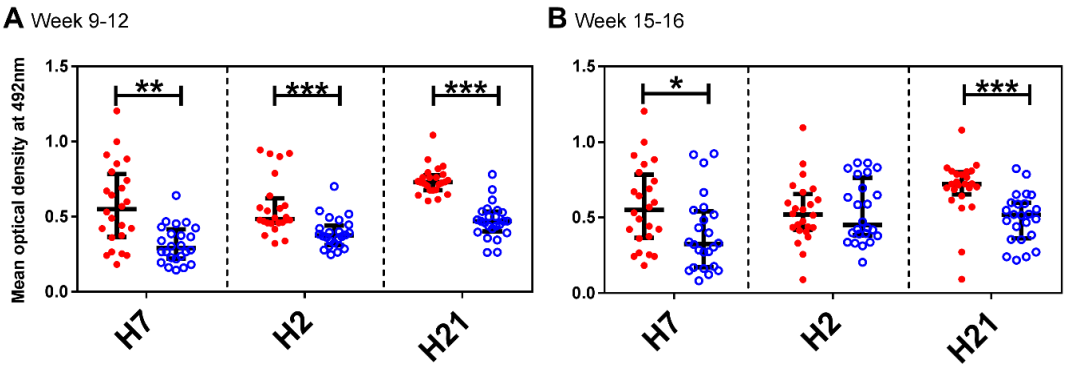


Figure 15: Flagellar specific IgG_1 responses in vaccinated and control calves in the toxoid study. ELISA plates were coated with H2, H21 or H7 corresponding to the STEC strains most commonly circulating in the group of calves during study. Each symbol represents the mean OD of duplicate determinations of diluted serum from one animal. The red solid symbols are the Stx toxoid vaccinated calves and the blue clear circles the control placebo vaccinated calves. The middle line represents the median and the error bars above and below the interquartile range. (A) Represents serum samples taken at 9-12 weeks; and (B) week 15-16. Statistical significance was determined using a Mann-Whitney U test. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Given the similar impact of Shiga toxoid vaccination on the IgG_1 response to the three different flagellins, correlation analysis was performed on H2, H21 and H7 responses (Figure 16). Significant positive correlations were found between H2 and H7 IgG_1 specific antibody levels at week 9-12 ($p < 0.0001$, $r_s = 0.6161$) but not at week 15-16 ($p = 0.1892$, $r_s = 0.1978$); significant correlations were also found between H21 and H7 specific IgG_1

antibody levels at both week 9-12 and week 15-16 ($p < 0.0001$, $r_s = 0.6390$ and $p = 0.0035$, $r_s = 0.4132$ respectively) and between H21 and H2 IgG_1 specific antibody levels at week 9-12 ($p < 0.0001$, $r_s = 0.6761$) and week 15-16 ($p = 0.0005$, $r_s = 0.4846$).

Sequence alignments of H2, H27 and H21 are shown in Figure 17 and 18. There was 48.96 % similarity between the H21 and H7 amino acid sequence and 46.97 % similarity between the H2 and H7 amino acid sequence. When comparing the conserved domains there was 78.8 % similarity between the H2 and H7 amino acid sequences, 77 % similarity between H21 and H7; and 88.8 % similarity between H2 and H21 amino acid sequences.

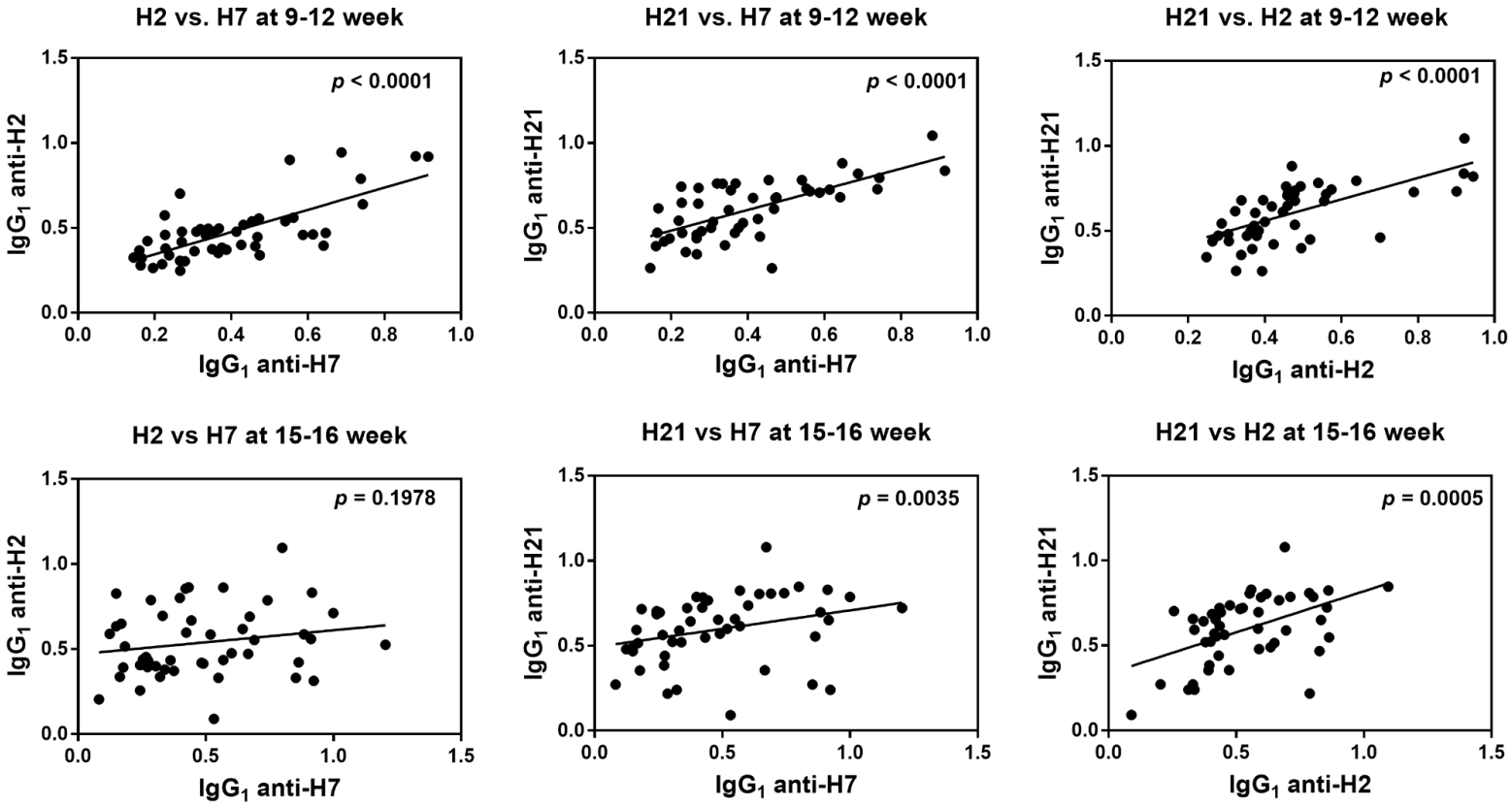


Figure 16: Correlations between H2, H7 and H7 specific IgG1 antibody responses in serum from the toxoid vaccination trial. Serum samples from 9-12 week and 15-16 week, ELISA results expressed as mean OD of duplicate wells, coats plated with H2, H7 or H21 protein preparations and antibodies used to determine IgG_1 antibody levels in serum samples. Each symbol represents an individual animal. *P*-values were determined using a Spearman's rank correlation coefficient.

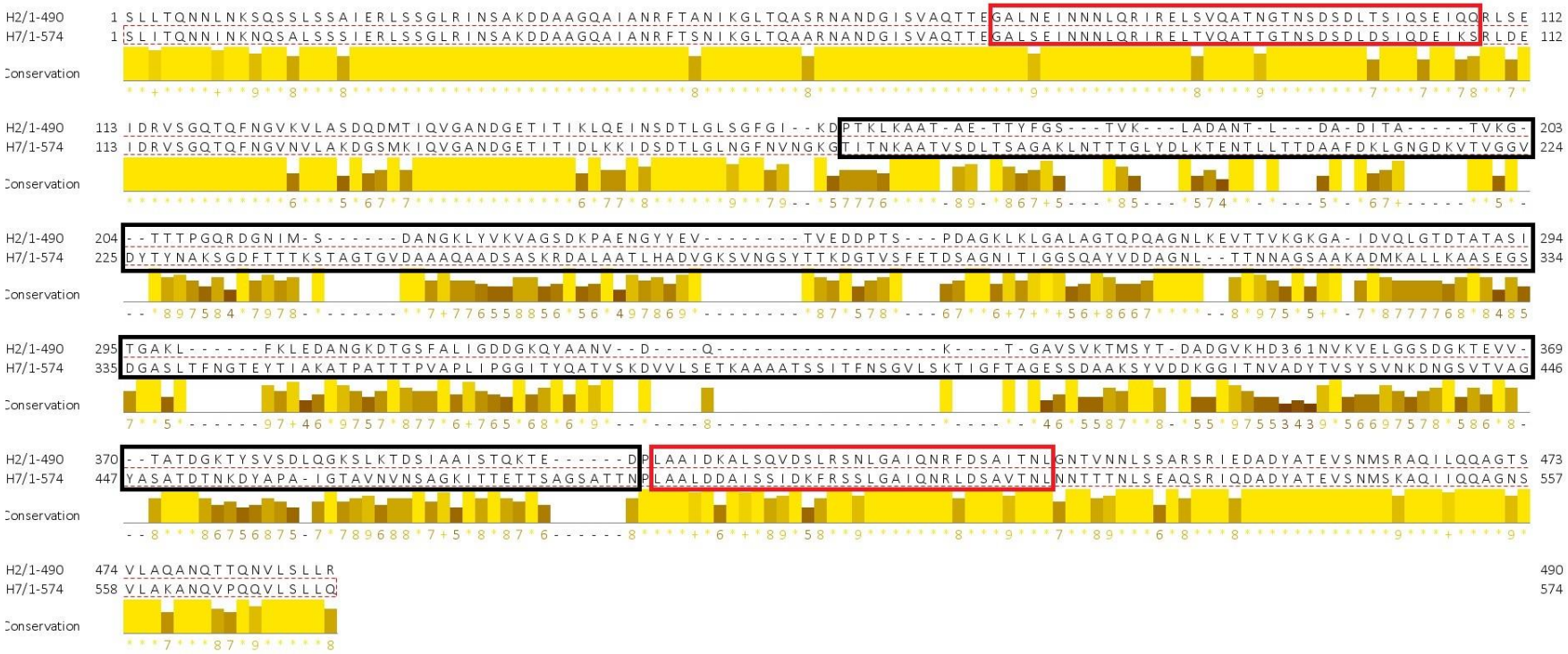


Figure 17: Aligned amino acid sequences of H2 and H7 flagellin, using Jalview (version 2.10.2)[162] to formulate the figure and compare the sequences. The black open boxes encapsulate the variable domains and the red open boxes encapsulate the putative TLR5 binding domains[161].

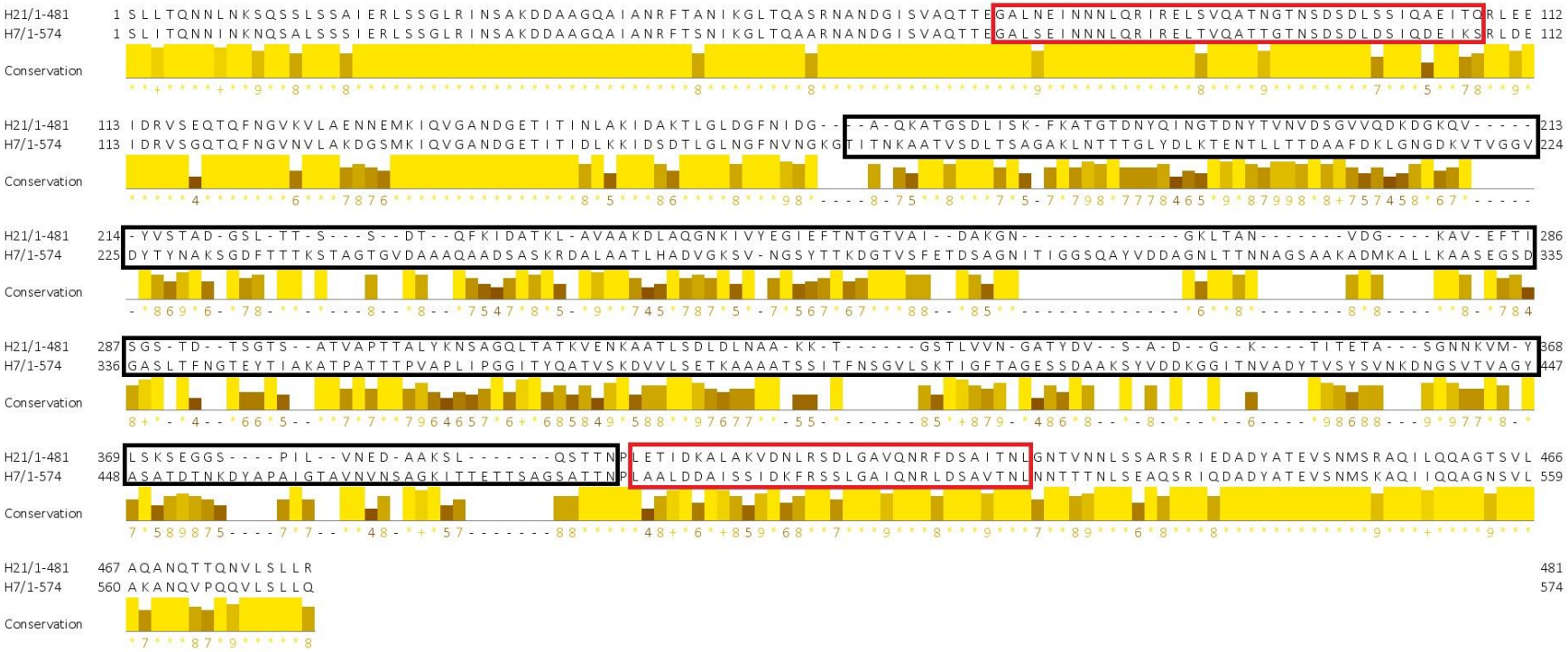


Figure 18: Aligned amino acid sequences of H21 and H7 flagellin using Jalview (version 2.10.2)[162], to formulate the figure and compare the sequences. The black open boxes encapsulate the variable domains and the red open boxes encapsulate the putative TLR5 binding domains[161].

2.3.7 Western blot analysis of flagellin-specific IgG_1 responses

Western blots were performed to investigate the specificity of serum IgG_1 antibodies for flagellin protein within the H2, H7 and H21 flagellin preparations. Serum samples were pooled from 5 animals that had high serum IgG_1 specific flagellin responses and another pool from 5 animals that had low serum IgG_1 specific flagellin responses. The western blot shown in Figure 19 demonstrate that IgG_1 antibodies bound to molecules of the predicted molecular weights for H2, H7 and H21 within the flagellin preparations (52 kDa, 60 kDa and 52 kDa for H2, H7 and H21, respectively). However, serum IgG_1 also bound to molecules of low molecular weight (approximately 6 kDa), particularly for H7 and H21 preparations. A stain for lipopolysaccharide (LPS) was performed on a SDS- PAGE gel of the three flagellin preparations to determine if the antibody-reactive low molecular weight molecule could be LPS. The results of the LPS staining indicate that all three flagellin preparations contained LPS, either at the same molecular weight as the flagellin protein or present at low molecular weights between 3 and 14 kDa. This suggests that some of the serum IgG_1 were binding LPS present within the flagellin preparations, particularly for the H7 and H21 preparations.

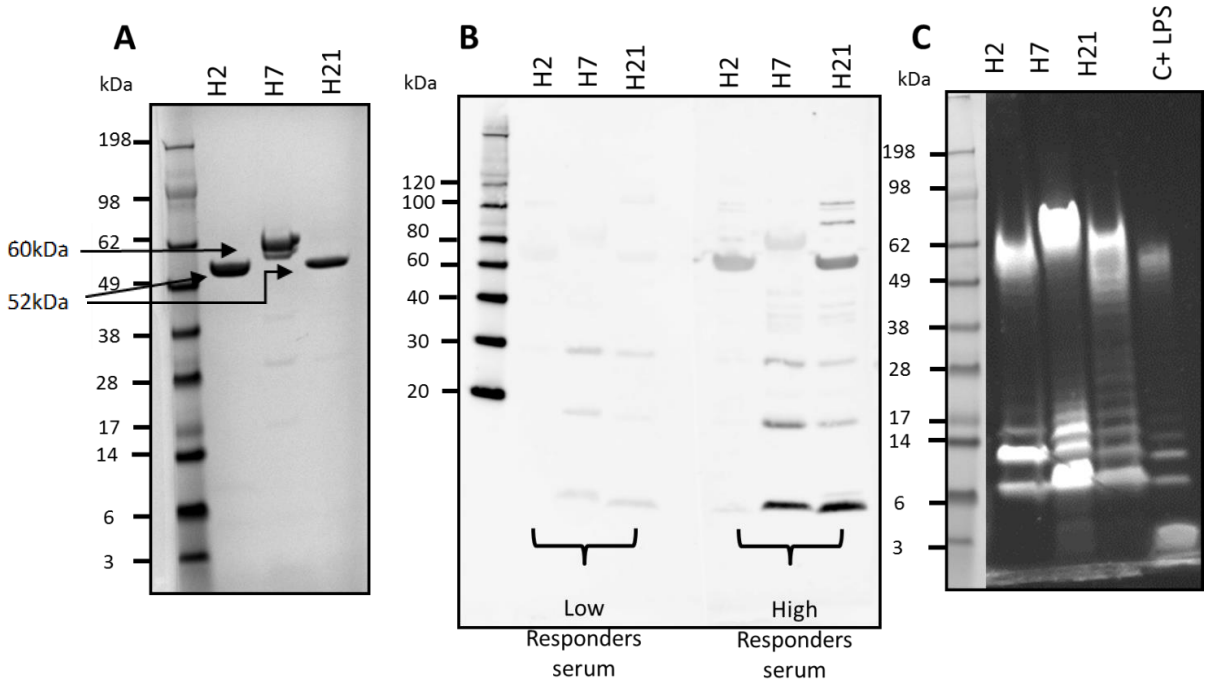
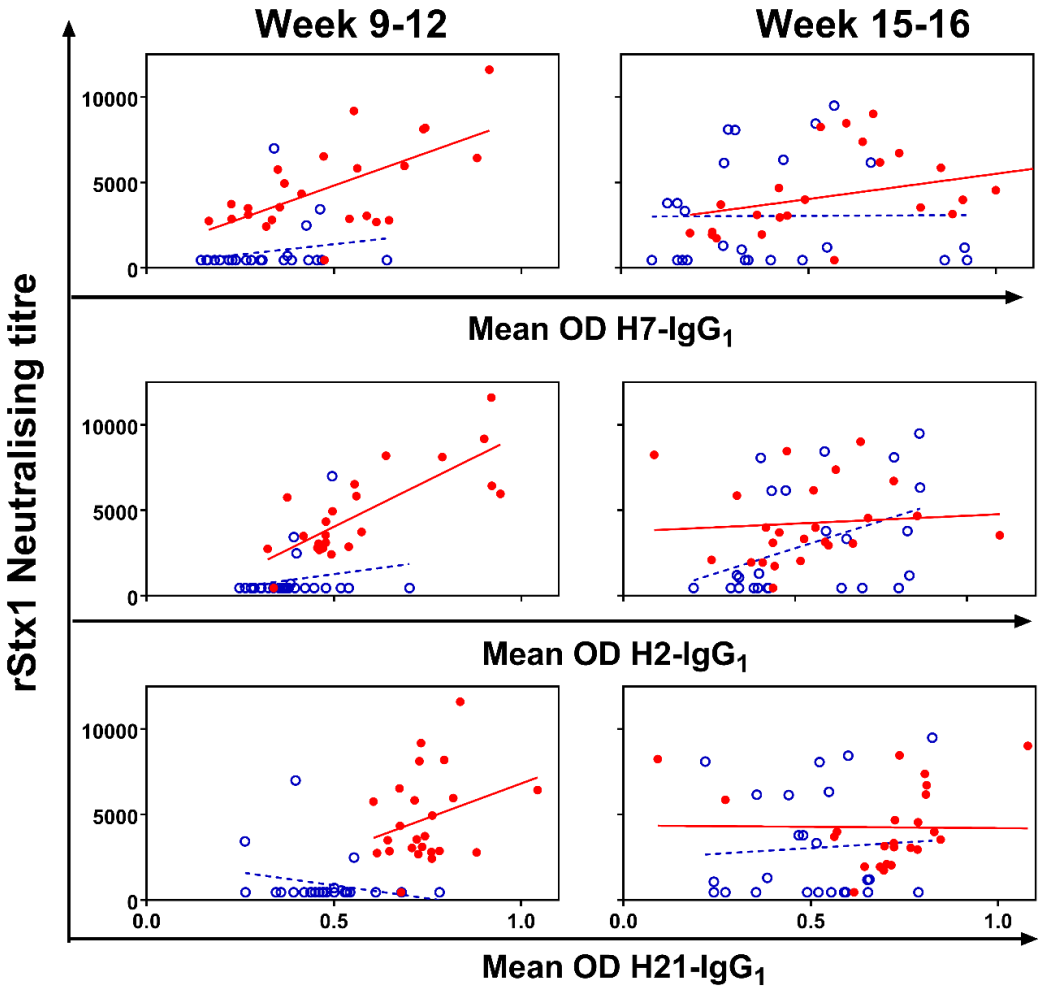


Figure 19: Characterisation of flagellin preparations by SDS-PAGE gel, western blotting and LPS staining. (A) Simply Blue™ (Invitrogen, Paisley, UK) stained SDS-PAGE gel of H2, H7 and H21 flagellin preparations together with a SeeBlue Plus2 (Invitrogen, Paisley, UK) protein standard (left-hand lane). (B) Western blot of H2, H7 and H21 preparations with pooled serum from calves with high or low levels of flagellin specific IgG_1 determined by ELISA to determine the specificity of the IgG_1 response. Following incubation with serum samples, the blot was incubated with a secondary antibody a mouse anti-bovine IgG_1 and then a tertiary rabbit anti-mouse IgG polyclonal antibody conjugated to HRP. (C) Staining of H2, H7 and H21 preparations separated by 1D SDS-PAGE using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Thermo Fisher Scientific, Loughborough, UK). C+ LPS = control LPS supplied with the kit.

2.3.8 Correlation between Stx neutralising antibody (nAb) titre and flagellin-specific antibodies

As we had hypothesised that neutralisation of Stx activity may enhance STEC-specific immunity upon natural STEC exposure, correlations between levels of Stx1 nAb titres and anti-flagellin IgG_1 responses were determined at the two time-points where Stx toxoid vaccination status coincided with increased flagellin-specific IgG_1. There were significant positive correlations between H7 specific IgG_1 and rStx1 nAb titres in vaccinated ($r_s = 0.497$, $p = 0.014$) but not control calves at 9-12 weeks ($r_s = 0.368$, $p = 0.077$; Figure 20) and also 15-16 weeks (vaccinated, $r_s = 0.519$, $p = 0.009$; control calves $r_s = 0.084$, $p = 0.697$). There were significant positive correlations between H2 specific IgG_1 and rStx1 neutralising antibody titres in vaccinated ($r_s = 0.752$, $p < 0.001$) but not control calves at 9-12 weeks ($r_s = 0.286$, $p = 0.176$). There were significant positive correlation between H2 specific IgG_1 and rStx1 neutralising antibody titres in control calves ($r_s = 0.406$, $p = 0.022$) but not in the vaccinated calves ($r_s = 0.308$, $p = 0.1434$) at 15-16 weeks. There were no significant correlations between H21 specific IgG_1 and rStx1 neutralising antibody titres (week 9-12, vaccinated calves $r_s = 0.225$, $p = 0.290$ and controls $r_s = 0.116$, $p = 0.599$; week 15-16, vaccinated calves $r_s = 0.351$, $p = 0.092$ and controls $r_s = -0.001$, $p = 0.999$).



		H7 specific IgG_1	H2 specific IgG_1	H21 specific IgG_1
rStx1 nAb titre	9-12 week	Toxoid vaccinated $r_s = 0.497$, $p = 0.014$	Toxoid vaccinated $r_s = 0.752$, $p < 0.001$	Toxoid vaccinated $r_s = 0.225$, $p = 0.290$
		Placebo controls $r_s = 0.368$, $p = 0.077$	Placebo controls $r_s = 0.286$, $p = 0.176$	Placebo controls $r_s = 0.116$, $p = 0.599$
	15-16 week	Toxoid vaccinated $r_s = 0.519$, $p = 0.009$	Toxoid vaccinated $r_s = 0.308$, $p = 0.143$	Toxoid vaccinated $r_s = 0.351$, $p = 0.092$
		Placebo controls $r_s = 0.084$, $p = 0.697$	Placebo controls $r_s = 0.406$, $p = 0.022$	Placebo controls $r_s = -0.001$, $p = 0.999$

Figure 20: rStx1 neutralizing titres compared to H7, H2 and H21 specific IgG_1 levels in serum in calves from the toxoid trial. H7, H2 and H21 specific IgG_1 antibody levels are expressed as the mean OD of duplicate determinations per animal normalised to a positive control sample. Red circles are toxoid vaccinated calves and blue open circles placebo vaccinated calves. *P*-values were determined by Spearman's rank correlation coefficient analysis and indicated in the table (correlation between rStx1 nAb titre compared to flagelin specific IgG_1).

2.4 Discussion

Shiga toxins are known immune modulators[62,136,139,153,163]. In the first part of this chapter we determined STEC antigen specific antibody levels in cattle naturally infected and shedding STEC O157. We showed that under field conditions, shedding of STEC O157 is associated with increased levels of H7-specific IgA and IgG_1 but levels of Tir-specific IgA are lower in cattle shedding moderate levels of STEC O157, suggesting at lower levels of colonisation the bacteria may be suppressing induction of Tir-specific humoral immunity. In the case of the other STEC O157 antigens investigated (Intimin and EspA) there was no evidence of an increase in antibody response to these antigens suggesting that these antigens are either poorly immunogenic or that the antibody response to these antigens was also suppressed. Although in this field study we do not know when the cattle were infected, for how long they were infected and if this was a primary or secondary infection. All of these factors may be contributing to the antibody responses seen. An alternative hypothesis that has been proposed is that colonisation with STEC sequesters IgA into the gut lumen, and this results in a reduction in levels of circulating STEC specific IgA[115]. However other studies have shown with gastrointestinal nematode infections (*Teladorsagia circumcincta)* that there is a good positive correlation between circulating and mucosal antigen-specific IgA[164], which does not support this alternative hypothesis.

Previous studies have shown that STEC O157 antigen antibody responses can be variable in field situations[165]. A study examining serum antibody levels found no significant difference between intimin and Tir antibody levels in STEC O157 negative compared to STEC O157 positive cattle farms. They also demonstrated that faecal secretion of STEC strains did not correlate with serum antibody responses to EspA, intimin or Tir[165]. The apparently different immunogenicity of H7 and other STEC antigens is potentially due to the T-cell dependency of the antigens, with H7 capable of acting as a T- cell independent antigen[166,167]. Thus, antibody responses to this antigen may be less sensitive to the effects of Stx, which is known to interfere with T-cell function both *in vitro*[136] and *in vivo*[126]. We are seeing H7 specific IgG_1 antibody production, so although H7 may be acting in a T-cell independent manner we are still seeing class switching of the antibodies which can occur with T-cell independent antigens[168,169]. A recent transcriptomic analyses of super shedding vs non-shedding cattle identified a suite of down-regulated transcripts in the terminal rectum of O157 colonised cattle associated with B-cell development[129] as discussed further in chapter 5. Interestingly at the time of rectal biopsy sampling in this study by Wang *et al.* (2016), most cattle were

actually shedding at low levels of STEC O157, and it may be that low levels of colonisation may be better at modulating host immunity as immune-modulators would be able to function in conditions of relatively low antigenic exposure. This may explain why suppression of Tir-specific antibody responses was only seen in cattle shedding moderate but not high levels of STEC O157. It is unlikely that the reduced levels of Tir-specific IgA are due to a general reduction in antibody levels rather than a specific regulation of Tir-specific B-cell response because we determined there was no statistically significant difference between total IgA, IgG_1 or IgG_2 antibody levels between animals in the cohorts in this study.

Based on these observations, and those of others which identify only weak and variable responses to STEC O157, we hypothesised that a significant component of this immunomodulatory effect was mediated by Stx, and that vaccination with a Stx toxoid would induce Stx neutralising antibodies which could interfere with this immune modulation and enhance antibody responses to STEC-specific antigens. Consistent with this hypothesis, our results indicate that Stx toxoid vaccines induce neutralising antibodies to Stx which correlate with significant reductions in STEC shedding but increased levels of flagellin-specific IgG_1. The strains of STEC found to be naturally circulating from calves in this study were H typed and the most common H types were found to be H21, H2 and H7 at week 15. Thus H7, H2 and H21 specific IgG_1 antibody levels were determined, and all showed to be significantly increased in calves that were vaccinated with the Stx toxoid at 9-12 weeks compared to control placebo vaccinated calves at the same time point. The flagellin-specific IgG_1 antibody levels were also found to highly correlate between the three H-types tested. This can be explained by the previous observation that anti-flagellin IgG_1 antibodies in cattle recognise epitopes in the N and C terminal domains of the protein which are conserved between different H-types[170,171].

One possible explanation for these results is that the toxoid vaccine preparation contained flagellin and/or LPS resulting in the induction of IgG_1 antibodies which bound to the flagellin preparations used for the ELISA tests which were shown to also contain LPS. Western blots confirm that the antibodies in the serum samples were binding to molecules of the same size as the flagellin proteins in the flagellin preparations; however, staining an equivalent gel also indicated that some of the antibody response may have been directed towards LPS within the flagellin preparations, particularly for H7 and H21. However the increased levels of H2, H7 and H21 specific IgG_1 seen in toxoid vaccinated calves compared to controls is unlikely to be due to vaccinated calves responding to flagellin and/or LPS

within the vaccine preparation for the following reasons: (i) FliC gene was absent from the parental strain used to generate the recombinant toxoid vaccine, and the strain was non-motile confirming a lack of flagellar expression[156], (ii) LPS was removed from the toxoid vaccine and (iii) the colostrum pools used as well as 24 hour serum samples from calves receiving colostrum from vaccinated dams contained significantly higher levels of Stx neutralising antibodies but similar levels of flagellin-specific IgG_1 compared to calves receiving colostrum from control dams. As post-colostral serum IgG_1 antibodies are reflective of the dam's IgG_1 response to the toxoid vaccine, there is no evidence to support the notion that the toxoid vaccine may have induced flagellin-specific IgG_1 antibodies itself. Instead, H-specific humoral immune responses detected in the groups of calves are most likely to result from natural exposure and development of an active immune response by the animals, with calves with high anti-Stx antibody titres better able to respond to antibody-mediated neutralisation of Stx mediated immune suppression.

Interestingly, while differences were seen between vaccinated and control calves in flagellin-specific antibodies, we saw no difference in response to the other antigens except at earlier time points (≤ 3 weeks post-colostrum) and Tir-specific IgA at 9-12 weeks. Differences in earlier time points reflect differences in colostral antibody levels rather than differences in the ability of the calves to respond immunologically to the antigens. The Tir-specific IgA responses were low for all calves in the trial and the significant difference between the groups marginal, being slightly higher in the control group at 9-12 weeks, possibly due to increased STEC exposure. The lack of observable differences in response to the other antigens may have been due to a lack of sensitivity to detect the differences: antibody responses were higher to flagellin preparations than to the other antigens, potentially making differences in flagellin-specific responses easier to detect. Whether this is due to the higher inherent immunogenicity of flagellins or higher exposure of the calves to flagellins, is unclear, although flagellin is a highly immunogenic molecule due to its ability to activate Toll-like receptor 5[172].

A toxoid vaccination that is able to lower STEC shedding in cattle to multiple serotypes of STEC could be of great economic and social consequence. Further work needs to be done to determine the use of this vaccine within the cattle population. If the vaccine leads to enhanced immune responses to STEC antigens, it could lead to enhanced immunogenicity of other non-O157 STEC strains. In conclusion, we provide evidence that STEC may modulate humoral immune responses in calves naturally colonised with moderate levels of STEC

O157. Furthermore, vaccination with Stx toxoids results in reduced STEC shedding but enhanced flagellin-specific antibody levels, suggesting that Stx may be involved in delaying or suppressing the development of humoral immunity in cattle.

Chapter 3

Immunity to STEC O157 following experimental challenge

3.1 Introduction

Following on from the natural colonisation chapter, we wanted to further define the immune responses in calves experimentally challenged with STEC O157. The group has previously developed an effective oral challenge protocol enabling successful STEC O157 colonisation of pre-weaned calves[97]. Using an oral experimental challenge protocol would allow us to use aged matched calves, challenged at the same time point with defined STEC O157 strains with known Stx repertoires.

Two different wild type strains were used for the STEC O157 calf challenges: a PT21/28 and a PT32 strain, both of which were *stx2c* positive. These strains were selected as they were representative of clinically relevant strains within the UK. A study in Scotland in 2003 investigating faecal pat samples from cattle found three main phage types of STEC O157; PT21/28 (46 %), PT32 (19 %) and PT8 (12 %)[30]. PT21/28 strains were more likely to be associated with high levels of shedding in cattle[30] compared to the other two PTs as indicated in Figure 21. Furthermore, PT21/28 stains were more likely to possess both *stx2a* and *stx2c* toxin subtypes[3], in contrast to PT32 strains which were predominately *stx2c*–positive alone[74]. The two strains used in this study were selected as they were representative of the PT21/28 and PT32 clusters determined by core-genome SNP analysis[173], had been isolated from faecal pats with relatively high (PT21/28) and low (PT32) bacterial counts, and possessed both Stx2a and Stx2c encoding prophages (PT21/28 strain) or Stx2c-encoding prophages alone (PT32 strain). The two strains had also been shown in a previous study to efficiently colonise calves following oral bolus challenge[97].

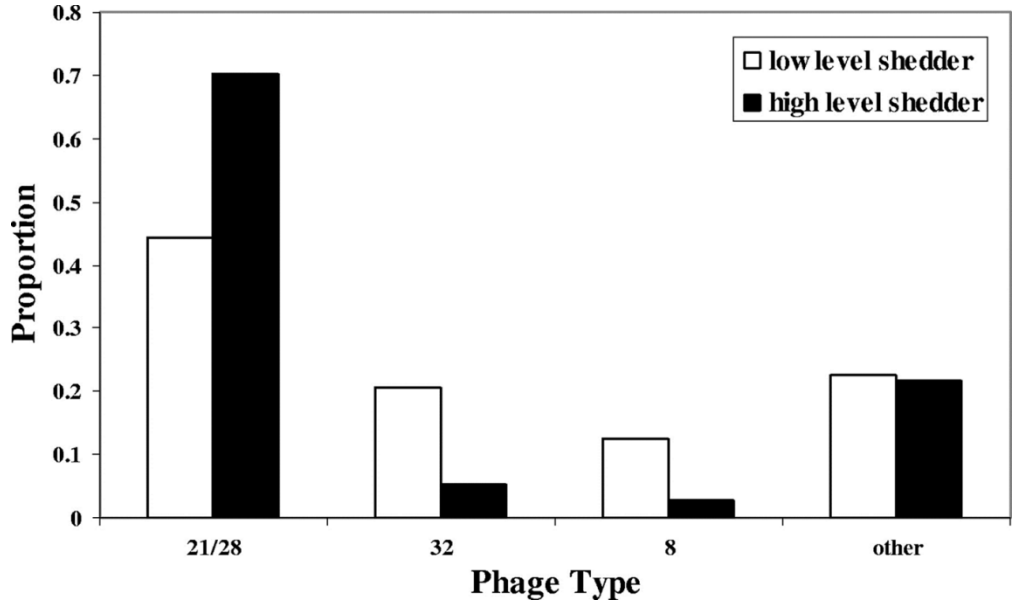


Figure 21: Comparison of proportions of the three major phage types (and others) for low-level and high-level shedders using the point estimate of the high-level threshold (≥ 3,135). Samples from Scottish farms taken March 2002 to February 2004; figure taken from Chase-Topping[30] with permission.

Through further sequence analysis it was found that the wild-type PT21/28 had an insertion sequence (ISec8) within the coding region of the A subunit of the *Stx2a* gene (Figure 22)[173], meaning that this strain was incapable of producing functional Stx2a toxin. The ISec8 was removed genetically enabling us to compare the effect on immune responses of two isogenic PT21/28 strains which differ only in the expression of a functional A subunit of the *Stx2a* gene (Fitzgerald *et al.* in preparation).

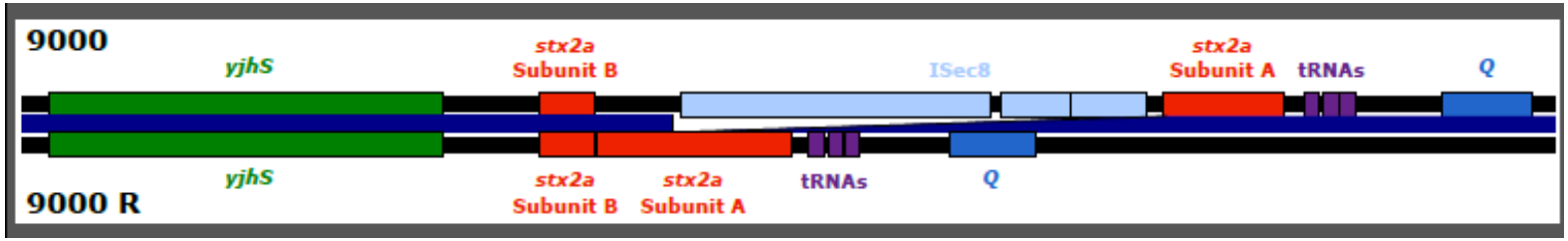


Figure 22: PT21/28 strain 9000 with the ISec8 insertion sequence (9000) compared to repaired isogenic PT21/28 strain (9000 R). The figure and sequencing information was generated by Sharif Shaaban (University of Edinburgh) using Easyfig alignment (version 2.2.2, company, Beatson Microbial Genomics Lab) that was then modified in Inkscape (version 0.91) for the labelling and the scaling of the image.

A recent study from within our group has provided evidence of cellular and humoral immune responses to type three secretion system (T3SS) proteins from STEC O157 in the rectal lymph nodes of calves colonised with the wild-type PT21/28 and PT32 strains[174]. Within the current study we wanted to investigate the systemic cellular and humoral immune responses in STEC O157 challenged calves to determine any strain-dependent differences in magnitude or timing of the response. Antibody responses as discussed in the previous chapter have been determined in a number of experimental challenge studies[115,175], but to our knowledge this is the first study that compares STEC O157 antigen specific antibodies in calves challenged with two strains differing only in their ability to produce functional Stx2a. The previous chapter demonstrated that a neutralisation of Stx activity (by toxoid vaccination) can lead to an increase in STEC specific antibody responses in a natural colonisation situation. We hypothesised that a challenge with STEC O157 (*stx* positive) strains would suppress the hosts STEC specific antibody responses, with the strain expressing both Stx2a+ and Stx2c+ been the most suppressive.

Main Aim

To quantify STEC-specific cellular and humoral responses following experimental challenge with STEC O157 strains expressing either Stx2a and Stx2c, or Stx2c alone.

3.2 Material and Methods

3.2.1 Type three secretion protein preparation

Type three secretion protein (T3SP) preparation was generated from *E. coli* O157 strain ZAP 193 (a *stx* negative strain). A single colony was used to inoculate 10 ml of LB (Luria broth) and incubated overnight at 37 ^{o}C (200 rpm). The 10 ml overnight culture was then added to 1 L of MEM-HEPES (Sigma-Aldrich, Dorset, UK) supplemented with 20 % (1 g/l) glucose, which was cultured at 37 ^{o}C (200 rpm) until reaching an OD 600 of 0.8. The bacteria were pelleted by centrifugation 5,000 *g* for 30 minutes and the supernatant subsequently filter sterilised using a 0.2 μm low protein binding filter (Milipore, Watford, UK). Trichloroacetic acid (TCA) at a final concentration of 10 % v/v was used to precipitate the protein overnight at 4 ^{o}C. The proteins were pelleted at 4 ^{o}C, 5,000 × *g* for 30 minutes. The pellet was re-suspended in 1.5 M Tris HCl pH 8.8. The preparation was than dialysed in phosphate buffered saline across a regenerated cellulose membrane which has a molecular weight cut-off rating of 3.5 kDa (Spectrum, labs, Breda, The Netherlands). Two batches of the above preparation were pooled at this stage and the protein content checked by separation using a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with SimplyBlue™ SafeStain (Invitrogen, Paisley, UK; Figure 23).

A bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA) was used to estimate the protein concentration in the final T3SP preparation. A EndoLISA kit (Hyglos, GmbH, Bernried, Germany) was used to estimate the lipopolysaccharide (LPS) concentration of the preparation, the plate was read using a Tecan microplate reader (Dynex Technologies, Worthing, UK) fitted with 380/10 nm excitation and 440/10 nm emission filters.

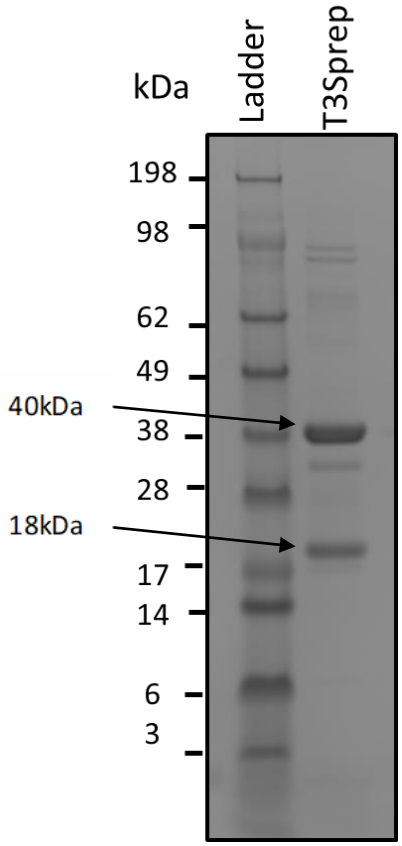


Figure 23: A 4-12 % w/v SDS-PAGE gel showing T3SP preparations used to determine antigen-specific recall responses in peripheral blood mononuclear cells (PBMC) and rectal lymph node cells. Migration of the SeeBlue Plus2 Pre-Stained standard ladder is indicated by tick and numbers on the left-hand side of the gel.

3.2.2 Experimental challenge trials

All animal work was carried out at the Moredun Research Institute (MRI) and ethical approval was obtained from the MRI animal experiments committee. All work was done under Home Office license. The animal trials were carried out as part of a larger scale STEC O157 shedding and transmission study (Fitzgerald *et al.* in preparation).

Conventionally reared male Holstein-Friesian dairy calves entered MRI at 3 weeks old and were pre-screened on a weekly basis 5 times before the oral challenge. Pre-screening involved obtaining faecal samples directly from the rectum from each calf and using IMS performed according to the manufacturer's instructions (Dynabeads anti-STEC O157;

Invitrogen, Paisley, UK) and subsequent culture confirming the calves to be STEC O157 negative when entering the trials and also by RT-qPCR (SERL protocol, performed by Dr Jason Morgan or Dr Stephen Fitzgerald) confirming the calves to be STEC O157 and *stx1* and *stx2* negative when entering the trials (Fitzgerald *et al*. in preparation).

The calves were randomly assigned into groups as detailed in Table 6. At this stage all of the calves were weaned. Calves were housed in rooms in the high security unit at MRI for challenge studies with the exception of no-challenge control calves which were conventionally housed (the group was housed separately from other animals on the farm) on the MRI farm.

Table 6: Details of STEC O157 experimental oral challenge trial protocol

Group	Challenge strain	Phage-type	Number of calves
PT21/28 Stx2c+ challenged	Strain 9000	PT21/28	6
PT21/28 Stx2c+ controls	None	n/a	5
PT32 Stx2c+ challenged	Strain 10671	PT32	6
PT32 Stx2c+ controls	None	n/a	4
PT21/28 Stx2a+Stx2c+ challenged	Strain 9000R	PT21/28	7
PT21/28 Stx2a+Stx2c+ controls	None	n/a	5

Calves were orally challenged by orogastric intubation with ~10^9 CFU of each strain within 10 ml of Lysogeny broth. For strain 9000, two of the six calves were challenged twice at a 7 day interval as the first challenge did not result in efficient colonisation. For the other strains, only one oral challenge was given.

3.2.3 Collection of faecal samples and bacterial counts

For orally challenged calves, faecal samples were collected by digital manipulation directly from the rectum daily for the first 18 days post oral challenge and then every other day. For control calves faecal samples were taken every other day. Ten gram of faeces were placed into 90 ml of sterile PBS. Ten-fold serial dilutions of the faecal samples were then prepared in PBS and 100 µl from three dilutions across a 1,000 fold range of dilutions were plated out in triplicate onto sorbitol MacConkey agar plates containing 15 µg nalidixic acid (NAL-SMAC). Plates were incubated at 37 ^{o}C overnight and colonies counted on the most suitable dilution plates. Randomly selected colonies from each plate were confirmed as O157 positive by using a latex agglutination kit (Oxoid, Basingstoke, United Kingdom) following the manufacturer's instructions. If no colonies were observed then overnight broth enrichment (tryptone soya broth [TSB; Oxoid, Basingstoke, USA]) from the undiluted faecal suspension was performed and then plated out on NAL-SMAC plates. Samples which were positive by broth enrichment were assigned a value of 10 CFU/g. Samples from the control animals were plated out directly onto cefixime –tellurite (CT)-SMAC plates containing 0.05 mg/L cefixime, 2.5 mg/L tellurite; incubated overnight at 37 ^{o}C and then enumerated the next day.

3.2.4 PBMC and serum preparations

Blood was collected weekly by jugular venepuncture for subsequent isolation of peripheral blood mononuclear cells (PBMC) and serum.

Serum was prepared by centrifugation of the blood at 330 *g* for 10 minutes and then the serum was aliquoted into 1.5 ml reaction tubes and stored at –20 ^{o}C prior to analysis.

PBMC were purified from heparinised blood samples by density gradient centrifugation using Ficoll-Paque (GE Health care life sciences, Little Chalfont, UK) in SepMate™ isolation tubes (STEMCELL Technologies, Inc., Vancouver, Canada) according to the manufacturer's instructions. Viable cells were counted using Trypan Blue viability stain (Sigma-Aldrich, Dorset, UK). PBMC were used immediately in the cellular assays.

3.2.5 Post Mortem and lymph node cell preparation

On day 25 or 26 of the trial calves were euthanized using intravenous pentobarbital. Rectal lymph nodes (draining the site of colonisation of STEC O157) were collected and placed in 35 ml transport medium (Hanks buffered saline solution [HBSS] without calcium and magnesium, 2 % heat-inactivated calf serum [HI-FCS], 10 mg/ml gentamicin [Sigma-Aldrich, Dorset, UK], 200 IU/ml penicillin, and 200 μg/ml streptomycin) prior to processing. Lymph nodes were washed twice in transport medium then placed into 15 ml preparation medium (transport medium without HI-FCS) and cut into small pieces using a scalpel blade. The lymph node and media was then transferred to a stomacher bag and placed in a stomacher (Colworth Stomacher 80, Seward Ltd, Worthing, UK) for 30 seconds. The content was then filtered through a 70 μm filter (Thermo-Fisher Scientific, Loughborough, UK) and the volume made up to 20 ml with transport medium. The suspension was then underlaid with 10 ml Ficoll-paque Plus (GE Health care Life Sciences, Little Chalfont, UK) and centrifuged for 30 minutes at 800 *g*. The mononuclear layer was harvested from the top of the Ficoll layer, washed twice with PBS and then re-suspended in complete culture medium (RPMI1640 [Gibco, Invitrogen, Paisley, UK], 10 % HI-FCS, 200 mM L-glutamine [Invitrogen, Paisley, UK], 0.004 % β-mercaptoethanol [Sigma-Aldrich, Dorset, UK], 100 IU/ml penicillin and 100 μg/ml streptomycin [Invitrogen, Paisley, UK]). Cells were either used for the generation of antibody secreting cell probes (see section below) or in lymphocyte stimulation assays.

3.2.6 Antibody Secreting Cell probe (ASC) generation

Antibody secreting cell probes were generated as previously described[176]. Briefly, lymph nodes cells (prepared as above) in complete culture medium were plated out (4 wells per lymph node) at 5×10^6 cells per well in 24 well plates to generate antibody secreting cell probes (ASC). The plates were incubated for 5 days at 37 ^{o}C and 5 % CO_2. For negative controls, complete culture medium without cells was incubated in parallel. After 5 days the cells were harvested from the plates into 15 ml conical centrifugation tubes (Falcon™, Thermo Fisher Scientific, Loughborough, UK), centrifuged at 330 *g* for 5 minutes and then the supernatant containing the secreted antibodies was collected and stored at -20 ^{o}C for future use.

3.2.7 Lymphocyte stimulation assays (LSA)

Cells (PBMC or lymph node cells) were seeded at 2 $\times 10^5$ cells per well into 96 well round bottom plates. Triplicate wells for each animal were stimulated with complete culture media (RPMI 1640 [Invitrogen, Paisley, UK] containing 10% Hi-FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol) containing either PBS, T3SP preparation (10 µg/ml), media containing a matched concentration of commercial STEC O111:B4 LPS (Sigma-Aldrich, Dorset, UK) to that present in the T3SP preparation or Concanavalin A (ConA; 10 µg/ml, Sigma-Aldrich, Dorset, UK) as a positive control. Plates were incubated for 4 days at 37 ^{o}C at 5 % CO_2. At day 4, 50 µl of supernatant was removed from each well and stored for at -20 ^{o}C for subsequent cytokine analysis. Fifty microliters of complete culture medium containing 0.5 µCi/well [3^{H}] thymidine (PerkinElmer, Cambridge, UK) was then added per well and the cells incubated for a further 18 hours. Cells were harvested onto glassfiber filters (PerkinElmer, Cambridge, UK) and [3^{H}] thymidine incorporation was quantified using an automated scintillation counter (PerkinElmer, Cambridge, UK). Stimulation indices were calculated by dividing the mean of the triplicate counts per minute by the mean of the relevant LPS stimulated controls.

3.2.8 Bovine IFN-γ ELISpot

A commercially available kit was used (ELISpot for Bovine IFN-γ; 3119-2HW-plus; MabTech, Nacka strand, Sweden) with hydrophobic polyvinylidene difluoride (PVDF) membrane ELISpot plates (Merck Millipore, Nottingham, UK) following the manufactures instructions. PBMC were stimulated with complete culture media containing either PBS, T3SP preparation (10 µg/ml), media containing a matched concentration of commercial STEC O111:B4 LPS (Sigma-Aldrich, Dorset, UK) or ConA (10 µg/ml) (Sigma-Aldrich, Dorset, UK) for 18 hours before addition to the plate in duplicate wells at 2 $\times 10^5$ cells per well. Reactive spots were developed using ELISpot TMB substrate and the number of spots was quantified automatically using an ELISpot reader (AID ELISpot reader version 4, Autoimmun Diagnostika GmbH, Strasberg, Germany). Responses of LPS-stimulated cultures were subtracted from responses by parallel T3SP stimulated cultures prior to data analysis. The results were expressed as spot forming units (SFU) per 1 $\times 10^6$ cells.

3.2.9 Quantification of antigen-specific antibody responses

Antigen specific IgA, IgG_1 and IgG_2 responses to H7, Tir, EspA and Intimin were quantified by indirect ELISA as described in Chapter 2. The optimum serum sample dilutions are shown in Table 7 and were determined following serial dilution of representative samples from each group to ensure that the colour reaction at an optical density (OD) at 492 nm for the sample was on the linear part of the curve. ASC probe samples were analysed neat for all antibody isotypes.

Table 7: Dilutions of serum samples for STEC antigen specific antibody ELISAs

	Dilution of serum samples		
Antigen	**IgA**	**IgG_1**	**IgG_2**
H7	1 in 10	1 in 100	1 in 50
Tir	1 in 10	1 in 250	1 in 50
EspA	1 in 10	1 in 50	1 in 50
Intimin	1 in 10	1 in 100	1 in 50

3.2.10 Statistical Analysis

Non-parametric Mann-Whitney *U* tests were used to compare rectal lymph node cells responses to *ex vivo* stimulation by T3SP between challenged and unchallenged control calves. STEC-antigen specific antibody responses in ASC probes between challenged and unchallenged calves were also compared using Mann-Whitney *U* tests. GraphPad prism (version 7.0, La Jolla, USA) was used for the statistical analysis above.

Mixed models were used to analyse temporal immune response data. All mixed model statistical analysis was performed using R (version 10) by Javier Palarea (Biomathematics and Statistics; Scotland; BioSS). The data was analysed separately for each STEC O157

strain. The T3SP-specific lymphocyte stimulation index data from PBMC was analysed by log transforming the T3SP-specific simulation index, then a generalised additive model with identity link function and Gaussian errors was fitted by restriction maximum likelihood (REML) to allow investigation of the effects of treatment (STEC O157 challenge vs. no challenge) on simulation index over the duration of the experiment. It included treatment group as fixed effect and spline-based smooth terms (one per treatment) to account for potential non-linear relationship of the response with time. Animal was added as a random effect and heterogeneous variances by treatment was allowed.

ELISpot (T3SP-specifc IFN-γ releasing cells) data was analysed by log transformation of the T3SP-specific spot forming units per 10^6 +1. A random interaction mixed model including treatment, time and their interaction as fixed effect and animal as random effect was fitted using restricted maximum likelihood (REML). An exponential within group variance structure over time was considered to model non-homogenous variances.

Generalised additive mixed models with identity link function and Gaussian errors were fitted by REML to investigate the effect of treatment on the STEC antigen specific antibody responses over time. The antibody responses were (log +1) transformed to normalise the data. The models included treatment group as fixed effect and spline-based smooth terms (one per treatment) to account for potential non-linear relationships of the response with time. Animal was added as random effect. Heterogeneous variances by group were allowed.

For all statistical tests a *p*-value of < 0.05 was considered significant.

3.3 Results

3.3.1 Faecal shedding

Figure 24 represents the individual shedding curves for animals orally challenged with STEC O157. The unchallenged control calves were negative for STEC O157 for the duration of the trial (data not shown). The shedding data confirms that all orally challenged calves were successfully colonised following their oral bolus challenge.

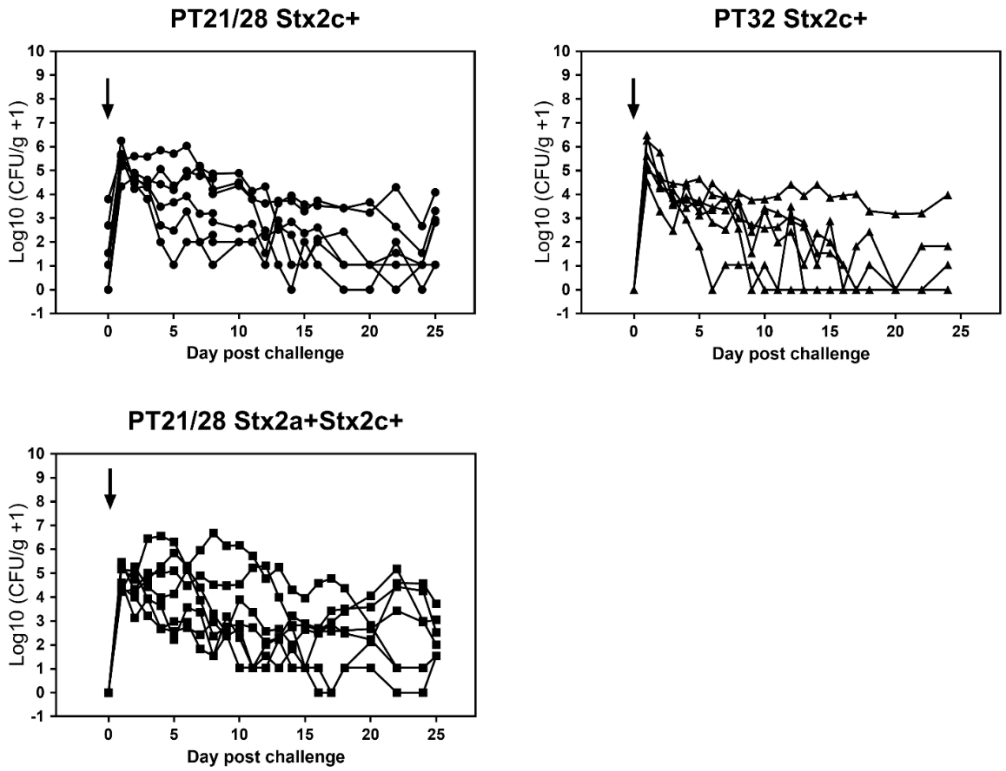


Figure 24: Faecal shedding curves of STEC O157 by experimental challenge of weaned calves. Six or seven calves in each trial were orally challenged with STEC O157 (PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+) indicated by the black arrow. For strain 9000 (PT21/28 Stx2c+), two of the six calves were challenged twice at a 7 day interval as the first challenge did not result in efficient colonisation. Curves represent shedding after successful challenge and were aligned relative to the challenge day. The shedding data is expressed as log10 of colony forming units per gram of faeces + 1. Each curve represents an individual animal.

3.3.2 Cellular immune responses

Figure 25 demonstrates T3SP-specific systemic cellular immune responses in calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+. PBMC from calves challenged with PT21/28 Stx2c+ showed no statistically significant difference in T3SP-specific lymphocyte proliferation ($p = 0.2592$) or in the number of T3SP-specific IFN-γ releasing cells ($p = 0.428$) to PBMC of unchallenged control calves. PBMC from calves challenged with PT32 Stx2c+ showed no statistically significant difference in T3SP-specific lymphocyte proliferation ($p = 0.8655$) or in T3SP-specific IFN-γ releasing cells between challenged and unchallenged control ($p = 0.469$). PBMC from calves challenged with PT21/28 Stx2a+Stx2c+ showed no statistically significant difference in T3SP-specific lymphocyte proliferation between challenged and unchallenged controls ($p = 0.895$). Numbers of T3SP-specific IFN-γ releasing cells were not determined for calves challenged with this strain as there were no statistically significant differences seen following challenge with the other two STEC O157 strains. The results suggest that oral challenge with all three STEC O157 strains induces a limited systemic cellular immune response to the T3SP preparations within the observation period.

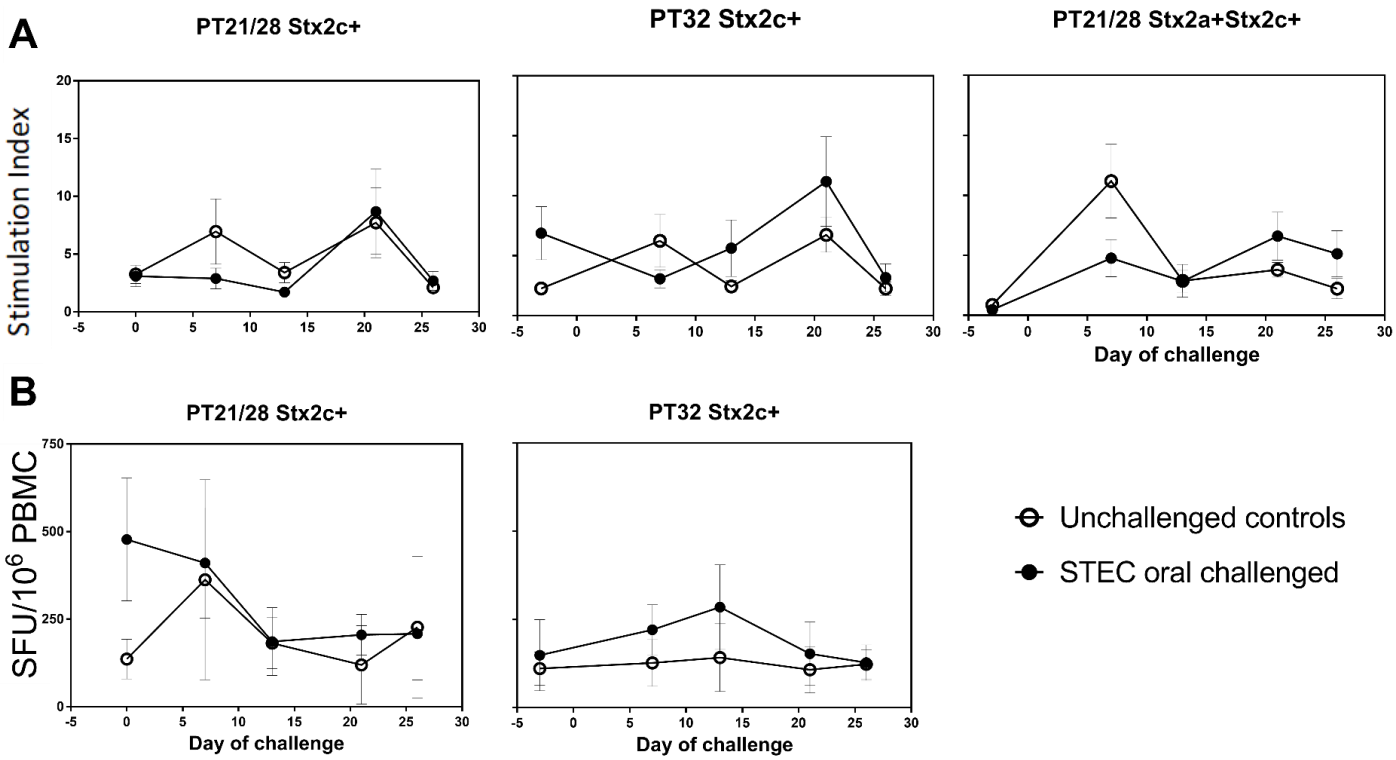


Figure 25: T3SP-specific lymphocyte proliferation and bovine IFN-γ spot forming units in PBMC prepared at weekly time points and stimulated *ex vivo* with T3SP preparation or LPS controls throughout the PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ trials. Proliferation (A) determined by a lymphocyte stimulation assay is expressed as indices, representing fold changes in the response to T3SP from levels with the relevant LPS control. Bovine IFN-γ releasing cells were determined by Elispot (B) and expressed as spot forming units per 10^6 cells in response to T3SP, responses of cultures stimulated with an equivalent amount of LPS to that present in the T3SP were subtracted before the data was analysed. Circles represent the mean of the group and error bars the standard error of the mean (SEM).

Antigen specific responses to a mucosal pathogen may be difficult to detect within PBMC unless samples are taken that coincide with recirculation of lymphocyte populations induced at the mucosal site of infection[177,178]. Thus the rectal lymph nodes were removed at post mortem from calves challenged with the two PT21/28 strains to capture the immune response in the draining lymph nodes to the primary site of colonisation of STEC O157 in bovines[27], as the lymph nodes would be expected to be enriched for STEC O157-antigen specific T-cells. However unlike PBMC the rectal lymph nodes cannot be used to study the temporal nature of the immune response as they are only accessible at post mortem (one time point). Rectal lymph node cells were re-stimulated *ex vivo* with the T3SP preparation and the lymphoproliferative response determined. The results are shown in Figure 26. For the PT21/28 Stx2c+ strain there was no statistically significant difference in rectal lymph node cell proliferation between the challenged and unchallenged control calves (p = 0.8030). Similarly for the PT21/28 Stx2a+Stx2c+ strain no significant difference in proliferation was observed between the challenged and unchallenged control calves (p = 0.6389).

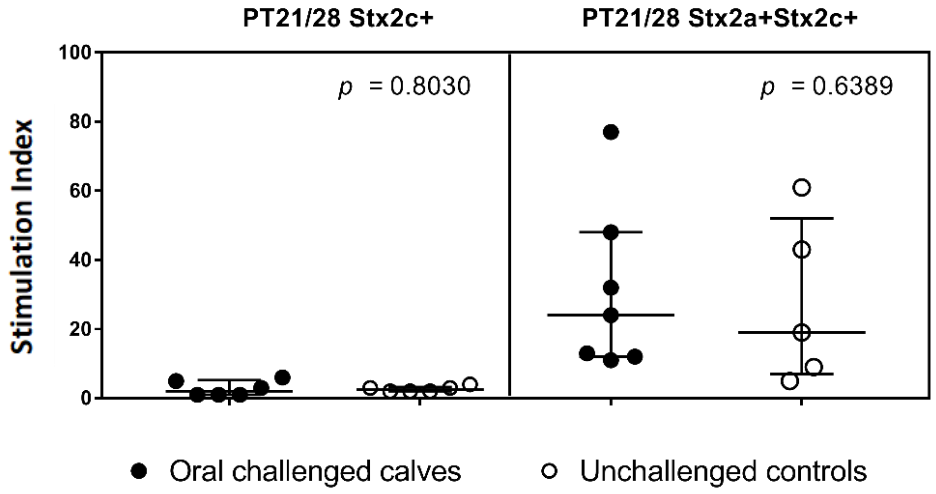


Figure 26: T3SP-specific proliferation of bovine lymph node cells isolated post-mortem from the rectal lymph node and re-stimulated *ex vivo* for 5 days with T3SP. Calves were orally challenged with either the PT21/28 Stx2c+ strain or the PT21/28 Stx2a+Stx2c+ strain. T3SP-specific proliferation was determined by a lymphocyte stimulation assay and is expressed as indices, representing fold changes in the response to T3SP relative to those from LPS stimulated control cultures. Circles represent individual calves and error bars represent median and interquartile range (IQR) of the group. The *p*-value is determined from a Mann-Whitney *U* test for differences between challenged and unchallenged calves for each strain.

3.3.3 Rectal STEC-specific antibody responses

Rectal lymph node ASC probes were generated to enable antibody responses local to the site of colonisation to be analysed. The results are shown in Figures 27 and 28. Levels of H7-specific IgA and IgG_1 were significantly higher in rectal ASC probes from calves challenged with the PT21/28 Stx2a+Stx2c+ strain compared to their unchallenged controls (Figure 27, $p = 0.025$ and $p = 0.025$ for IgA and IgG_1, respectively). In contrast, levels of H7-specific IgA or IgG_1 within ASC probes from PT21/28 Stx2c+ or PT32 Stx2c+ challenged calves were not significantly different from their unchallenged controls. There were no statistically significant differences in the levels of H7-specific IgG_2 between challenged and unchallenged calves with any of the three STEC O157 strains.

Levels of Tir- specific IgA were significantly higher in rectal ASC probes from calves challenged with the PT21/28 Stx2a+Stx2c+ strain ($p = 0.018$), but not the PT21/28 Stx2c+ or PT32 Stx2c+ strains, compared to their respective unchallenged controls (Figure 27). No statistically significant differences in the levels of Tir-specific IgG_1 or IgG_2 were observed between challenged and unchallenged calves for any of the three STEC O157 strains.

Levels of EspA-specific IgA were significantly higher in rectal ASC probes from calves challenged with the PT21/28 Stx2a+Stx2c+ strain ($p = 0.012$), but not the PT21/28 Stx2c+ or PT32 Stx2c+ strains, compared to their respective unchallenged controls (Figure 28). No statistically significant differences in the levels of EspA-specific IgG_1 or IgG_2 were observed between challenged and unchallenged calves for any of the three STEC O157 strains.

Levels of intimin- specific IgA were significantly higher in rectal ASC probes from calves challenged with the PT21/28 Stx2a+Stx2c+ strain ($p = 0.018$), but not the PT21/28 Stx2c+ or PT32 Stx2c+ strains, compared to their respective unchallenged controls (Figure 28). No statistically significant differences in the levels of intimin-specific IgG_1 or IgG_2 were observed between challenged and unchallenged calves for any of the three STEC O157 strains.

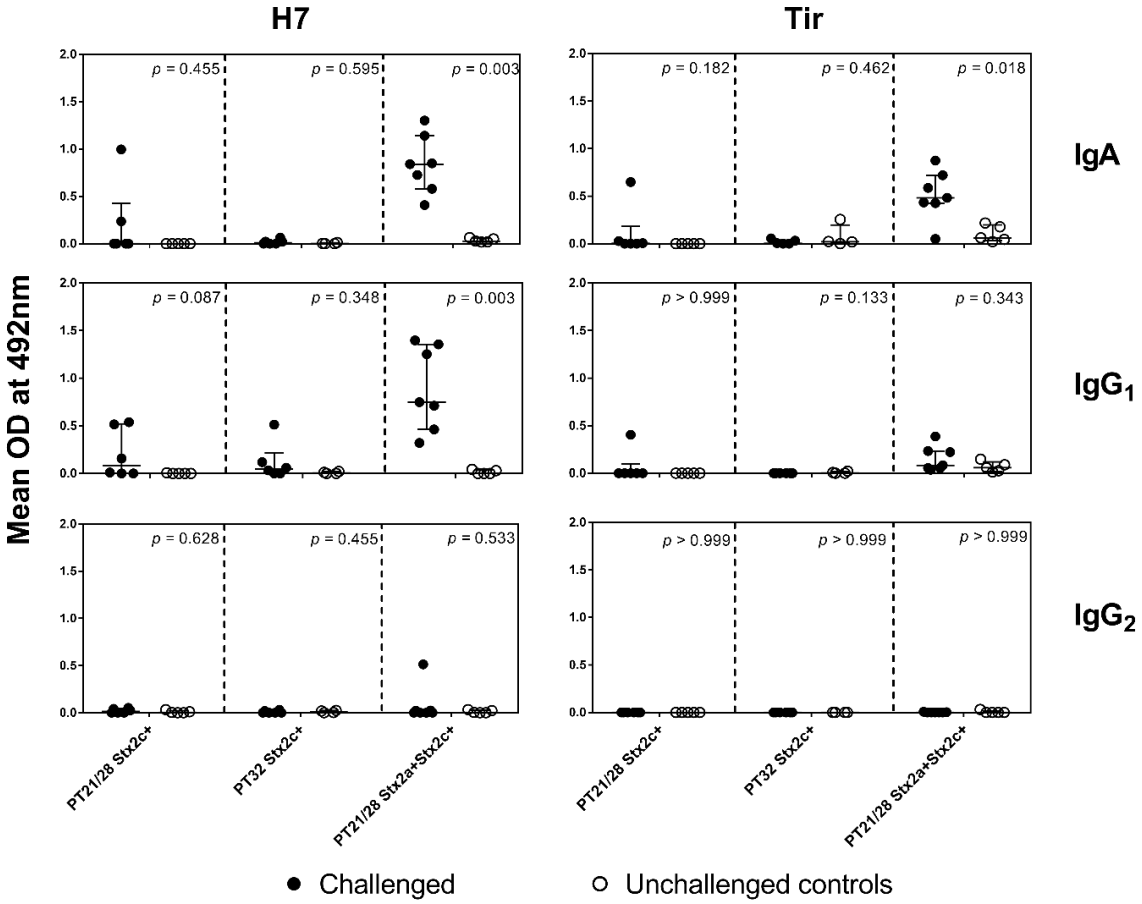


Figure 27: Levels of H7-and Tir-specific antibodies within rectal lymph node antibody secreting cell probes (ASC) generated from STEC O157 challenged and unchallenged calves. ASC probes were generated from rectal lymph nodes of calves challenged with PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2c+Stx2a+ and their relevant unchallenged controls. Levels of H7- and Tir-specific antibodies were determined by ELISA. The error bars represent medians and interquartile ranges. Each symbol represents an individual animal. Mann Whitney *U*-tests were used to compare antibody levels between challenged and unchallenged control calves for each strain and associated *p*-values are indicated.

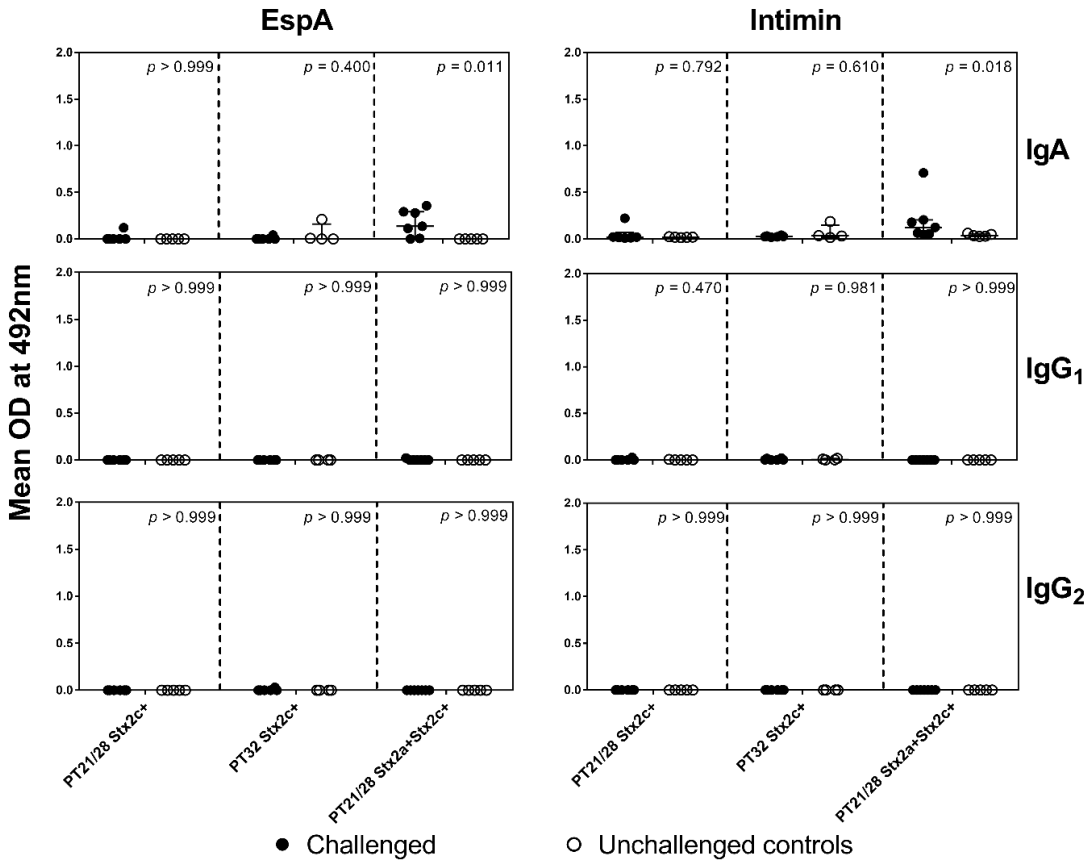


Figure 28: Levels of EspA- and intimin-specific antibodies within rectal lymph node ASC probes generated from STEC O157 challenged and unchallenged calves. ASC probes were generated from rectal lymph nodes of calves challenged with PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2c+Stx2a+ and their relevant unchallenged controls. Levels of EspA- and intimin-specific antibodies were determined by ELISA. The error bars represent medians and interquartile ranges. Each symbol represents an individual animal. Mann Whitney *U*-tests were used to compare antibody levels between challenged and control calves for each strain and associated *p*-values are indicated.

3.3.4 Systemic STEC-specific antibody responses

The systemic antibody responses to H7, Tir, EspA and intimin were assessed by quantifying levels of antigen-specific antibodies within serum samples collected at multiple time points throughout the trial. Levels were compared between challenged and their respective unchallenged control calves for each of the three STEC O157 strains. Serum levels of H7-specific antibodies are shown in Figure 29. Circulating levels of H7-specific IgA were significantly higher in calves challenged with all three STEC O157 strains compared to their unchallenged controls ($p = 0.002$, $p < 0.001$ and $p < 0.001$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively). Serum H7-specific IgG_1 responses were significantly higher in calves challenged with the PT21/28 Stx2a+Stx2c+ strain ($p = 0.029$), but not the PT21/28 Stx2c+ ($p = 0.075$) or PT32 Stx2c+ strains ($p = 0.060$), compared to their unchallenged controls. No significant differences in serum H7-specific IgG_2 responses were found for any of the three strains ($p = 0.967$, $p = 0.128$ and $p = 0.056$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively).

Serum levels of Tir-specific antibodies are shown in Figure 30. No significant differences in serum Tir-specific IgA responses were found for any of the three strains ($p = 0.517$, $p = 0.440$ and $p = 0.299$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively). Serum Tir-specific IgG_1 responses were significantly higher in calves challenged with the PT32 Stx2c+ strain ($p = 0.045$), but not the PT21/28 Stx2c+ ($p = 0.599$) or PT21/28 Stx2a+Stx2c+ ($p = 0.419$) strains, compared to their unchallenged controls. No significant differences in serum H7-specific IgG_2 responses were found for any of the three strains ($p = 0.639$, $p = 0.909$ and $p = 0.321$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively).

Serum levels of EspA-specific antibodies are shown in Figure 31. No significant differences in serum EspA-specific IgA responses were found for any of the three strains ($p = 0.143$, $p = 0.585$ and $p = 0.988$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively). Serum EspA-specific IgG_1 responses were significantly higher in calves challenged with the PT32 Stx2c+ strain ($p = 0.023$), but not the PT21/28 Stx2c+ ($p = 0.501$) or PT21/28 Stx2a+Stx2c+ ($p = 0.056$) strains, compared to their unchallenged controls. No significant differences in serum H7-specific IgG_2 responses were found for any of the three strains ($p = 0.173$, $p = 0.328$ and $p = 0.367$ for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively).

Serum levels of intimin-specific antibodies are shown in Figure 32. No significant differences in serum intimin-specific IgA responses were found for any of the three strains (p = 0.089, p = 0.882 and p = 0.563 for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively). No significant differences in serum intimin-specific IgG_1 responses were found for any of the three strains (p = 0.074, p = 0.555 and p = 0.981 for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively). Finally, no significant differences in serum intimin-specific IgG_2 responses were found for any of the three strains (p = 0.430, p = 0.266 and p = 0.978 for P21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+, respectively).

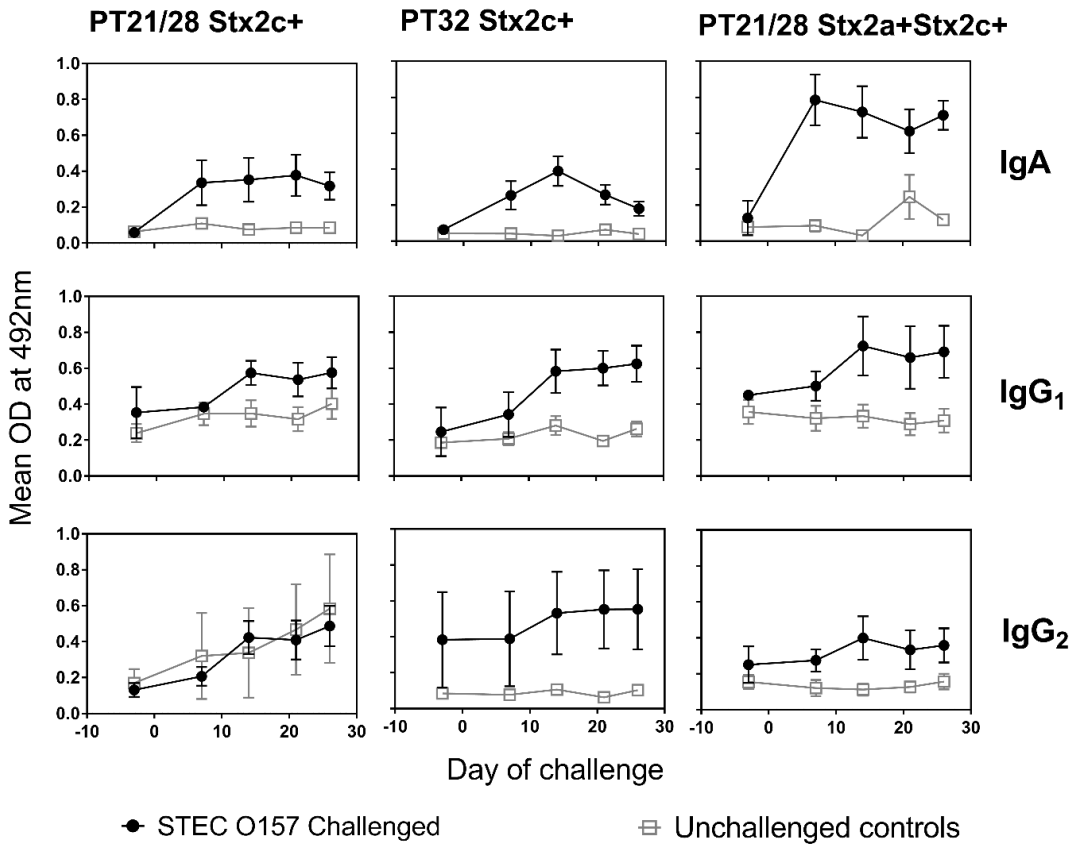


Figure 29: Serum levels of H7-specific antibodies in STEC challenged and unchallenged calves. IgA, IgG_1 and IgG_2 antibody levels in serum were determined using an ELISA from serum collected at weekly time points from calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ and their unchallenged controls calves. Each symbol represents the mean of each groups and the error bars the SEM. The H7-specific antibody levels are expressed as the mean OD of duplicate wells normalised to a positive control sample.

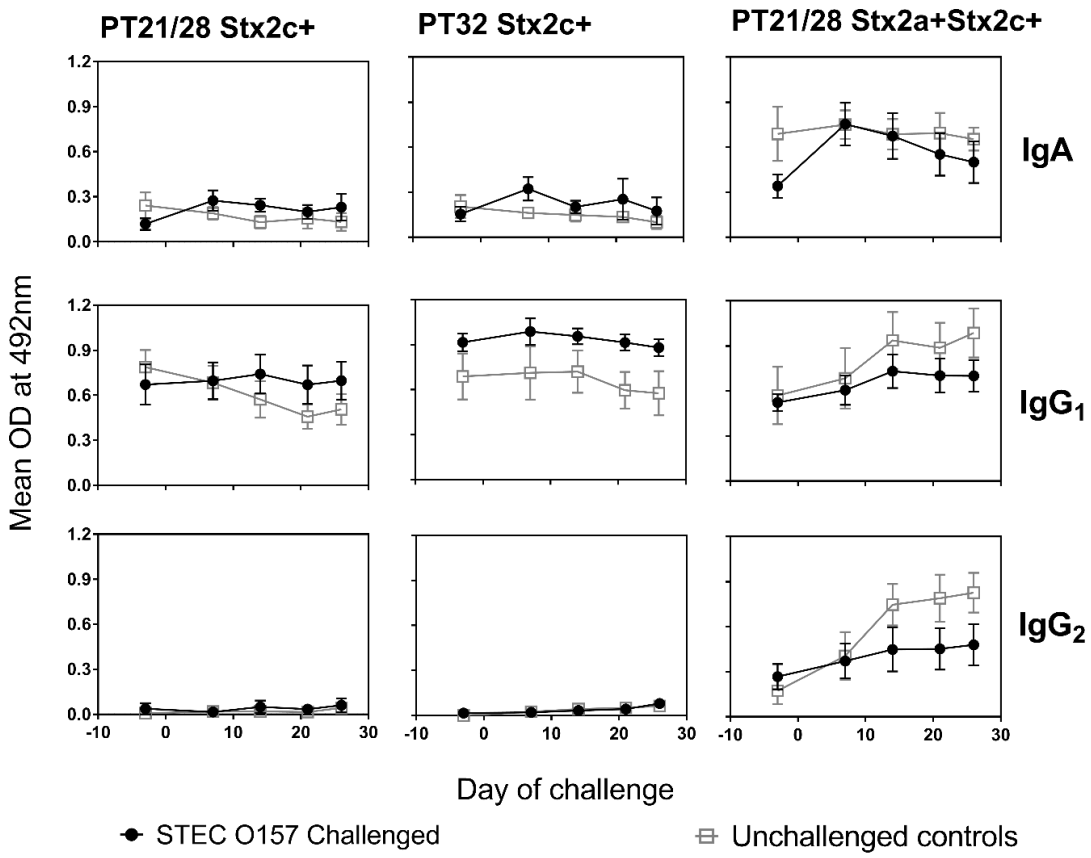


Figure 30: Serum levels of Tir-specific antibodies in STEC challenged and unchallenged calves. IgA, IgG_1 and IgG_2 antibody levels in serum were determined using an ELISA from serum collected at weekly time points from calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ and unchallenged controls calves. Each symbol represents the mean of each groups and the error bars the SEM. The Tir-specific antibody levels are expressed as the mean OD of duplicate wells normalised to a positive control sample.

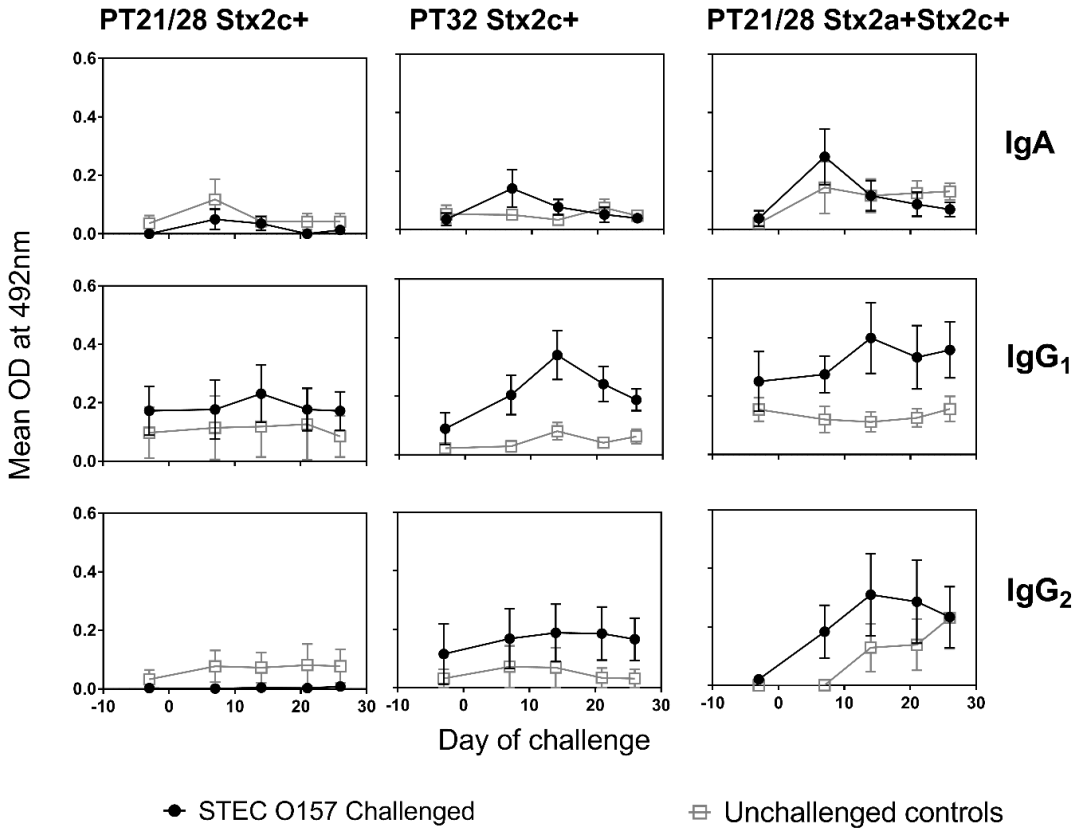


Figure 31: Serum levels of EspA-specific antibodies in STEC challenged and unchallenged calves. IgA, IgG_1 and IgG_2 antibody levels in serum were determined using an ELISA from serum collected at weekly time points from calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ and unchallenged controls calves. Each symbol represents the mean of each groups and the error bars the SEM. The EspA-specific antibody levels are expressed as the mean OD of duplicate wells normalised to a positive control sample.

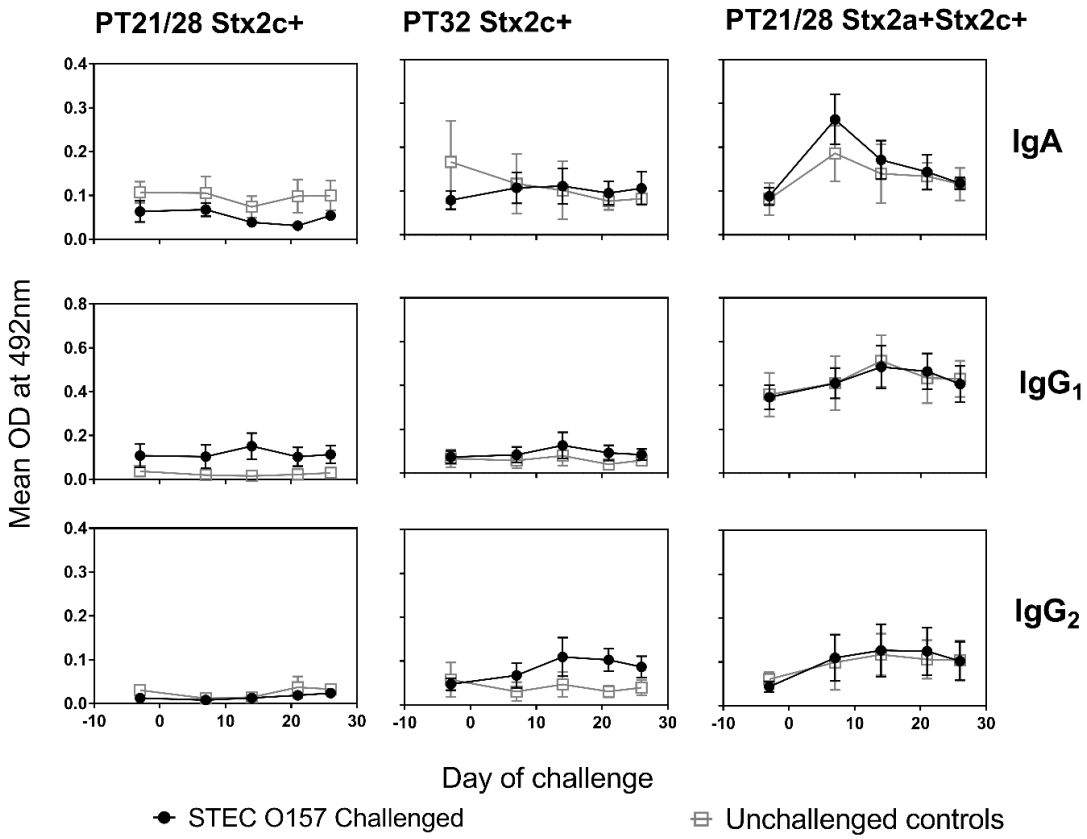


Figure 32: Serum levels of Intimin-specific antibodies in STEC challenged and unchallenged calves. IgA, IgG_1 and IgG_2 antibody levels in serum were determined using an ELISA from serum collected at weekly time points from calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ and unchallenged controls calves. Each symbol represents the mean of each groups and the error bars the SEM. The Intimin-specific antibody levels are expressed as the mean OD of duplicate wells normalised to a positive control sample.

3.4 Discussion

To understand the field study results described in Chapter 2 in more detail experimental animal trials were performed to assess STEC O157-specific responses in more defined STEC O157 orally challenged animals. The experimental animal trials were performed as part of a larger experiment studying the shedding and transmission dynamics of three STEC O157 strains PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ in cattle (Fitzgerald *et al*. in preparation) and this enabled variation in host cellular immune responses and antibody responses between the three strains to be analysed.

To identify the presence of circulating adaptive immune responses following STEC O157 colonisation PBMC preparations were prepared, pre-challenge and then weekly following oral STEC O157 challenge and stimulated *ex vivo* with an STEC O157 T3SP preparation which had previously been shown to contain epitopes recognised by mucosal helper ($CD4^+$) T-cells from STEC O157 challenged calves[179]. Lymphocyte activation was determined by assessing antigen-specific proliferation and as a previous study in the group had determined that STEC O157 colonisation was associated with a local T-helper (T_H) Type 1 response[174], ELISpot assays were used to determine the number of antigen-specific IFN-γ producing cells within the PBMC population. The effects of contaminating LPS within the T3SP preparation was controlled for by using parallel LPS stimulated cultures in these experiments. In addition to analysing circulating cellular immune responses, for the two PT21/28 strains lymphoproliferative responses to the T3SP preparation within the rectal lymph node, which drains the site of STEC O157 colonisation[27], were also analysed. However, no significant differences in either local or systemic T-cell specific lymphoproliferative responses, or numbers of antigen-specific IFN-γ producing cells, were found between challenged and unchallenged control groups for any of the three strains tested. All the calves included in the study were found not to shed STEC O157 when screened during the 5 weeks immediately prior to challenge. However, calves may have been previously exposed to some of the T3SP antigens as calves may start shedding STEC strains even in the first weeks of life when raised on a conventional farm[160]. It is also possible that the frequency of antigen-specific T-cells within the circulation may have been too low to detect any differences between challenged and control calves, although this would not explain the failure of rectal lymph node cells to respond to the T3SP preparation as it would be expected that a higher frequency of STEC-specific T-cells would be present in the rectal lymph node. This suggests

that STEC O157 colonisation induces a limited cellular immune response, which would be consistent with work done previously within the group which also failed to detect differences in rectal lymphoproliferative responses to T3SP between challenged and control calves[174].

Comparative levels of IgA, IgG_1 and IgG_2 specific to the STEC antigens (H7 flagellin, Tir, EspA and intimin) were determined in serum samples from calves orally challenged with PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ STEC O157 strains. As determined with serum samples from naturally infected cattle, cattle challenged with STEC O157 (and shedding high levels of STEC O157) showed a statistically significant increase in systemic H7-specific IgA levels compared to negative control animals. All three challenge strains lead to a significant increase in systemic H7-specific IgA.

Systemic H7-specific IgA antibody levels increased 7 days post challenge and peaked for PT21/28 Stx2a+Stx2c+ challenged calves although staying relatively high until the end of the trial. H7-IgA peaked at 21 days for PT21/28 Stx2c+ challenged calves and 14 days for PT32 Stx2c+ challenged calves. The fairly rapid decline in systemic H7-specific IgA may be explained by the relatively short half-life of IgA[180]. Bretschneider *et al.* (2007) also showed a similar decline in STEC antigen specific IgA shortly after challenge and proposed this may be evidence of their use in response to challenge through binding of STEC O157 and its secretory proteins[115]. As stated previously there was significant difference in mean bacterial shedding counts due to the distinct behaviour of the PT32 Stx2c+ strain challenged calves, there was a more rapid decline in their mean shedding over time; so by the end of the trial there were lower levels of PT32 Stx2c+ shed in the faeces (Fitzgerald *et al.* in preparation). This would fit in with the more rapid decline in H7-specific IgA in the PT32 Stx2c+ challenged calves.

The systemic H7-specific IgG_1 and IgG_2 responses were more variable and two challenged calves had high H7-specific IgG_1 responses pre-challenge. There was a significant difference in the H7-specific IgG_1 response with both the PT32 Stx2c+ and PT21/28 Stx2a+ Stx2c+ challenge strains. Tir-, EspA- and intimin-specific antibody levels in serum were more variable within the groups. Some animals had relatively high levels of Tir-specific IgA, IgG_1 and IgG_2 levels prior to challenge with STEC O157. EspA- and intimin-specific antibody levels were lower in all animals compared to Tir and H7. Asper *et al.* (2011) have previously determined significant levels of EspA-specific antibody levels in serum in two experimentally challenged animals[175]. STEC-specific IgG_2 systemic antibody levels were generally lower than IgG_1 in all calves and this agrees with previous studies[181].

STEC O157 in cattle infects a mucosal surface and peripheral immune responses in sera do not always reflect the local immune responses[182]. Antibody secreting cells (ASC) probes have been used previously to study the local immune response to a number of pathogens resident at mucosal surfaces[176,183,184]. ASC probes were used to allow assessment of the local antibody response to STEC O157 colonisation (rectal lymph node probes). The H7-specific IgA in the rectal lymph node ASC probes were only statistically significantly different with the PT21/28 Stx2a+Stx2c+ strain challenge calves compared to their controls, unlike the systemic responses (statistically significant difference with all three strains). The only difference between the PT21/28 Stx2c+ and PT21/28 Stx2a+Stx2c+ strains is the removal of the ISec8 insertion sequence and thus the ability of the strains to produce an active A subunit of Stx2a. Given the hypothesis that Stx suppress bovine immune responses is true and that the latter strain produces both Stx2a and Stx2c it may be expected that the toxins act in an additive manner and PT21/28 Stx2a+Stx2c+ challenged calves would have lower H7-specific IgA responses rather than higher ones as observed in this study. The data presented does not support this assumption. This however may not reflect what is occurring in natural exposure in the field. The experimental challenge is a single large bolus of bacteria, we only challenged with two wild type strains and also the calves were all young with developing immune systems. These factors and others maybe effecting the immune responses seen in this study.

The magnitude of the antibody response may partly reflect the level of antigen challenge[185]. A different group has previously demonstrated differences in systemic EspA-specific IgA responses between calves orally challenged with 10^{10} compared to 10^{8} CFU of bacteria[181]. Statistical analysis of the STEC O157 faecal shedding data (Fitzgerald *et al.* in preparation) has determined that there was a statistically significant difference in mean counts over time between the three strains ($p = 0.006$). Further pairwise tests of mean differences between strains support that the statistically significant difference in mean counts is mostly explained by the distinct behaviour of the PT32 strain and not between PT21/28 Stx2c+ and PT21/28 Stx2a+Stx2c+ (Fitzgerald *et al.* in preparation). Thus it is less likely that the amount of antigen could be affecting the H7-specific IgA responses, unless the strains differ in their surface expression of flagellin or the flagellin in the different strains differ in their immunogenicity.

There were significant differences in local H7-specific IgG_1, Tir-specific IgA and EspA-specific IgA responses between PT21/28 Stx2a+Stx2c+ challenged calves and their unchallenged control calves. It is not known if flagellin responses tend to be higher than

other STEC antigens because flagellin is more immunogenic (of the STEC antigens tested it is the only one which can directly activate an innate immune receptor) or because it is less affected by any immunosuppressive effects of Stx. In this study the PT21/28 Stx2a+Stx2c+ challenged calves had significantly higher H7-specific IgA and IgG_1 local antibody responses, whereas the PT21/28 Stx2c+ and PT32 Stx2c+ challenged calves did not, so it seems unlikely that Stx are having a significant suppressive effect on H7 responses in these calves. Again with the Tir-specific IgA responses there were significantly higher Tir-specific IgA responses in the PT21/28 Stx2a+Stx2c+ challenged calves but not in the PT21/28 Stx2c+ or PT32 Stx2c+ challenged calves. The PT21/28 Stx2a+Stx2c+ strain produces an active Stx2a+ as well as Stx2c+ unlike the other two challenge strains used which just produce Stx2c+. Data from within our group has shown that *in vivo* the PT21/28 Stx2a+Stx2c+ produces more Stx than the other two challenge strains (Dr Stephen Fitzgerald, personal communication). Also in Vero cell assays Stx2a has been shown to be more than 25 times more potent than Stx2c and also *in vivo* studies in mice showed Stx2a to be more potent than Stxc[186].

All IgG_2 antibody levels in the rectal lymph node generated ASC probes were lower than IgA or IgG_1. Intimin- and EspA-specific IgA and IgG_1 were also lower than against Tir and H7. This data concurs with previous data from within the group and other studies indicating that colonisation with a Stx positive STEC O157 often leads to only a poor mucosal antibody response[174]. The detection of sporadic local IgA levels again agrees with previous studies[121]. This may reflect immune modulation by Stx at this local site, however one may have expected lower antibody responses in calves challenged with the PT21/28 Stx2a+Stx2c+ strain possessing active Stx2a and Stx2c genes compared to the other two strains just possessing active Stx2c+ genes, and the PT21/28 Stx2a+Stx2c+ strain has been shown to produce higher levels of Stx2 *in vitro* (Dr Stephen Fitzgerald, personal communication).

The data from these experimental oral challenge animal trials indicate that oral challenge with STEC O157 lead to limited cellular immune responses to T3SP but some induction of humoral responses, both systemically and local to the site of STEC O157 colonisation. The PT21/28 Stx2a+Stx2c+ strain generally leads to more significant STEC antigen specific antibody responses. This indicates that although there is weak cellular responses there is no evidence that a functional Stx2a+ leads to an increased level of immune suppression. It has been previously shown in a mouse model that Stx can lead to upregulation of nucleolin[154]. Nucleolin is a eukaryotic protein, recognised by intimin and associated with sites of STEC attachment[187]. In the same mouse model it was also demonstrated that a *stx* positive strain

E. coli O157 colonised at a higher level than a *stx2* isogenic mutant *E. coli* O157 strain and also adhered better to epithelial cells in tissue culture[154]. This mouse study indicates that Stx may be able to promote colonisation in the short term, this would lead to enhanced T-cell independent and B-cell immunity perhaps before T-cell inhibition can become apparent. In this experimental study, if the calves were pre-immune to T3SP as indicated in the cellular assays, then they may be less sensitive to Stx immune suppression. The colonisation promoting effect may have outpaced the immunosuppressive effect at this stage and in this experimental setting.

The major limitation of this study is that we were not able to compare the results with calves that were challenged with a Stx negative strain. As discussed in the introduction chapter, STEC O157 produce a number of effector proteins with the potential to modulate the host immune response. The direct comparison between an isogenic *stx* negative strain and *stx* positive strain would have allowed us to make more definite conclusions regarding the specific effect of Stx on the STEC-specific adaptive immune response in cattle.

Chapter 4

STEC O157 effect on a bystander immunisation

4.1 Introduction

Vaccinations are an important tool in livestock production. In many vaccine studies evidence of variation in host response have been reported[188-190], with a varying proportion of non-responders even after several vaccinations[191,192]. Much of this variation is believed to be related to the genotype of the host, with particular emphasis on the major histocompatibility complex (MHC) which plays a central role in antigen presentation to T-cells[193]. Other non-genetic factors could influence an animal's ability to respond to vaccinations such as on-going malnutrition and co-infections. Helminths have developed complex mechanisms to suppress and/ or avoid the mammalian immune response[98,194-196] and to enable long-term survival within these hosts. Helminth mediated immune modulation can affect responses to parasite specific but also to non-parasite specific antigens (so called 'bystander' immune suppression). As a consequence, helminth co-infections have been associated with poor vaccine efficacy[196-200]. Other studies in cattle have demonstrated that although concurrent helminth infections can reduce cytokine responses to respiratory vaccinations there was no significant effect of helminth infections on the antibody responses to respiratory vaccinations[201,202]. However in other livestock species including pigs and chickens, helminth infections have been shown to suppress antibody levels to concurrent vaccinations[200,203]. A study has shown that malaria infections in mice can compromise established adaptive immunity to a live, attenuated vaccine against *Salmonella Typhimurium*[204]. The malaria infection had no effect on antibody responses to the vaccine but did suppress CD4 and CD8 T-cell effector responses and lead to an increase in IL-10 expression[204].

There is little information in the literature about concurrent bacterial infections and vaccine performance. One study showed that the quantity of campylobacter and enterovirus at the time of administration of primary oral polio vaccination in children in Bangladesh has been associated with reduced polio neutralizing titers[205]. As previously described in the introduction, STEC O157 colonisation has been associated with down-regulation of a

number of genes involved in adaptive immune responses within the intestinal mucosa[97], and can produce a number of proteins with *in vitro* immunosuppressive activity[64,126,136,137,139,140,206], presumably with the main objective of enabling more efficient colonisation of the bovine intestinal epithelium. Stx are likely to circulate throughout the body and thus Stx has the potential to modulate immune responses systemically as well as locally. Whether 'bystander' immune suppression occurs with STEC O157 infections in cattle is currently unknown. STEC O157 is prevalent in many cattle populations. Therefore if STEC O157 colonisation has a negative impact on the ability to respond to vaccinations or concurrent infections, this could have far-reaching effects on cattle health and welfare.

The aim of this chapter was to determine if STEC O157 colonisation affects the ability of cattle to respond to a concurrent immunisation. Ovalbumin (OVA), a T-cell dependent antigen, it was selected as the antigen for immunisation as it has been widely used as a model antigen in cattle due to its ability to elicit robust antibody and cellular immune responses, and that it is not encountered in the natural environment of cattle[207-209]. Furthermore, OVA is antigenically distinct from STEC O157 antigens, being the main protein found in chicken eggs[207], and can be obtained in a highly pure form[210]. Therefore the immune response to OVA would be highly tractable. OVA immunisations were administered on two occasions, two weeks apart together with the saponin adjuvant Quil A. Saponins have been used in a variety of veterinary vaccines[211,212] and induce mixed T_H1/T_H2 immune responses[213,214], meaning that the effects of STEC O157 on modulating both T_H1 and T_H2 immunity could be assessed. The timing of the first immunisation was designed to coincide with peak STEC O157 shedding (five days post-challenge) as it has previously been shown that when evaluating STEC O157-specific immune responses, T-cell priming rather than recall of memory T-cells is most affected by Stx activity[126]. Cellular, cytokine and humoral responses to OVA were monitored and compared between STEC O157 challenged calves and calves which were not STEC O157 challenged.

Main Aim:

To determine the effects of STEC O157 colonisation on cellular and humoral immune responses to a non-STEC O157 T- cell dependent antigen and if any affect will vary with strains of STEC O157 with different Stx repertoires.

4.2 Material and Methods

4.2.1 Animal trials

Two calf trials were performed (Tables 8 and 9, also see chapter 3). Briefly calves were orally challenged with ~10^9 colony forming units (CFU) of the following STEC O157 strains: PT21/28 Stx2c+ strain 9000, PT32 Stx2c+ strain 10671 or PT21/28 Stx2a+Stx2c+ strain 9000R in Trial 1, and PT21/28 Stx2a+Stx2c+ strain 9000 only in Trial 2. Calves were immunised on two separate occasions (5 days and 19 days post-challenge) in the left hand side of the neck via the subcutaneous route with 60 μg low-endotoxin ovalbumin (EndoGrade® Ovalbumin, Hyglos GmbH, Bernried am Starnberger See, Germany) plus 5 mg Quil A (Brenntag Biosector, Frederikssund, Denmark). In Trial 1, two calves challenged with each of the three strains were challenged only and not OVA immunised.

Table 8: Details of experimental groups in Trial 1.

Group	Bacterial challenge	Total no. of calves	No. OVA immunised
PT21/28 Stx2c+ challenged and immunised (C-I)	Strain 9000	4	4
PT21/28 Stx2c+ challenged only (C)	Strain 9000	2	0
PT21/28 Stx2c+ controls (immunised only [I])	None	5	5
PT32 Stx2c+ challenged and immunised (C-I)	Strain 10671	4	4
PT32 Stx2c+ challenged only (C)	Strain 10671	2	0
PT32 Stx2c+ controls (immunised only [I])	None	4	4
PT21/28 Stx2a+Stx2c+ challenged and immunised (C-I)	Strain 9000R	5	5
PT21/28 Stx2a+Stx2c+ challenged only (C)	Strain 9000R	2	0
PT21/28 Stx2a+Stx2c+ controls (immunised only [I])	None	5	5

Table 9: Details of experimental groups in Trial 2.

Group	Bacterial challenge	Total no. of calves	No. OVA immunised
PT21/28 Stx2a+Stx2c+ challenged and immunised (C-I)	Strain 9000	6	6
PT21/28 Stx2a+Stx2c+ controls (immunised only [I])	None	6	6

4.2.2 Bacterial faecal shedding counts

Data was collected as described in chapter 3 and expressed as colony forming units per gram of faeces (CFU/g).

4.2.3 PBMC and serum preparations

Blood was collected by jugular venepuncture on days -3, 7, 13, 21, and 25/26 for subsequent isolation of peripheral blood mononuclear cells (PBMC) and serum. PBMC and serum was prepared as described in chapter 3.

4.2.4 Lymph node cell preparation

At post mortem pre-scapular lymph nodes (PsLN) from the left hand side (i.e. draining the site of immunisation) were removed and placed into 35 ml of preparation medium (Hank's balanced salt solution (HBSS) without calcium or magnesium, 2 % heat inactivated FCS, 10 mg/ml gentamicin (Sigma Aldrich, Dorset, UK), 200 IU/ml penicillin and 200 µg/ml streptomycin). Single cell suspensions were then prepared as detailed in Chapter 3.

Cells were then either used immediately for ELISpot and Lymphocyte stimulation assays, or cryopreserved by re-suspending in Hi-FCS containing 10 % (vol/vol) dimethylsulfoxide (DMSO; Sigma-Aldrich) and freezing in a CoolCell® FTS30 (BioCision, California, USA) before longer-term storage in liquid nitrogen.

4.2.5 Lymphocyte stimulation assays (LSA)

Assays were performed as described in chapter 3 except cells were stimulated with OVA (10 µg/ml), Concanavalin A (Con A; 10 µg/ml [Sigma-Aldrich]) or an equivalent volume of PBS (unstimulated control) in RPMI 1640 (Invitrogen) containing 10 % Hi-FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol for 4 days at 37 ^{o}C and 5 % CO_2. Stimulation indices were calculated by dividing the mean of

the triplicate CPM for each stimulant by the mean of the corresponding unstimulated (PBS) controls.

4.2.6 Antibody Secreting Cell probe (ASC) generation

Probes were generated as described in Chapter 3.

4.2.7 Quantification of OVA-specific antibody responses by Enzyme Linked Immunosorbent assay (ELISA)

Responses were determined following a protocol described previously[161] using antibodies listed in Table 10. All wash steps were 5 washes with wash buffer (PBS with 0.05 % Tween20) and all incubation steps were 1 hour at 37 ^{o}C unless stated otherwise. Immulon 2HB 96 well MicroTitre plates (Thermo Electron Corporation, Milford, USA) were coated with 50 µl low-endotoxin ovalbumin (EndoGrade® Ovalbumin, Hyglos GmbH, Bernried am Starnberger See, Germany) at 1 µg/ml diluted in 0.05 M carbonate buffer (1.59 g Na_2C0_3, 2.93 g $NaHCO_3$ made up to 1 L in dH_2O, pH 9.6) and incubated overnight at 4 ^{o}C. Plates were washed and then incubated with 100 µl of blocking buffer (PBS with 3 % fish gelatine [Sigma-Aldrich]) to reduce non-specific antibody binding. Plates were washed again and 50 µl of the samples were added to each well in the plate in duplicate as follows; ASC probes generated from PsLN cells were added to the plate undiluted; serum samples were added 1:100 for OVA- specific IgG_1 and 1:4 for OVA-specific IgG_2. The optimum sample dilutions were determined following serial dilution of representative samples from each group to ensure that the colour reaction at an optical density (OD) at 492 nm for the sample was on the linear part of the curve. Plates were incubated for 1 hour and then washed again; 50 µl of secondary antibodies (mouse anti-bovine IgA monoclonal antibody (mAb) (clone K84.2F9), anti-bovine IgG_1 mab (clone K37.2G6) or anti-bovine IgG_2 mab (clone K192.4F10) (all mabs from AbD Serotec, Oxford, UK) were added and again the plates were incubated for 1 hour. Plates were washed again, 50 µl of monoclonal rat anti-mouse IgG_1 conjugated to HRP (LO-MG1-2, AbD Serotec) was added and the plates incubated for 1 hour. Plates were washed and developed using 100 µl per a well of *o*-phenylenediamine dihydrochloride (OPD) and stopped after 12 minutes with 25 µl per well of stop solution (2.5M H_2SO_4). The plates were read at 492 nm using a Tecan plate reader (Dynex Technology, Worthing, UK).

The final OD was calculated by normalising the OD to the average plate blank control OD, and inter-plate variation was normalised to the OD of a positive control sample.

4.2.8 Cytokine ELISAs

Levels of OVA specific IFN-γ and IL-10 in supernatants collected from LSAs after 4 days of stimulation were quantified by ELISAs. All ELISAs were performed using Immulon 2HB 96 well MicroTitre plates (Thermo Electron Corporation, Milford, USA). All plates were read at 450 nm using the Tecan plate reader (Dynex Technology, Worthing, UK) and OVA specific response determined by removing background levels of cytokines determined in cells cultured in medium supplemented with PBS only. All samples were run in duplicate wells and a standard curve run on every plate. The details of the antibodies used in the cytokines ELISAs and their dilutions are in Table 10.

IFN-γ ELISAs

A commercially available kit was used (Bovine IFN-γ ELISA kit [HRP], 3119-1H-6, MabTech, Nacka strand, Sweden) following the manufacturer's instructions. Briefly, plates were coated with 50 µl of primary antibody (mouse anti-bovine IFN-γ; clone MT17.1) and incubated overnight at 4 ^{o}C. Plates were then washed, and incubated with 100 µl of wash buffer with 0.1 % BSA (incubation buffer) for 1 hour to reduce non-specific binding. Plates were washed again; samples (diluted 1 in 4, with incubation buffer) and standards (50 µl) were added to the plate and incubated for 2 hours. Plates were washed and 50 µl mouse anti-bovine IFN-γ (clone MT307b) was added to the plate and incubated for 1 hour. Plates were washed and then incubated with 50 µl of Streptavidin-HRP (MabTech, Nacka strand, Sweden) for 1 hour. Plates were washed and developed with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL SureBlue TMB peroxidase substrate, Insight technologies, London, UK); the reaction was stopped with 50 µl of TMB stop solution.

IL-10 ELISAs

Plates were coated with 50 µl of primary antibody (mouse anti-bovine IL-10; clone CC318) diluted in 0.05 M carbonate coating buffer and incubated overnight at 4 ^{o}C. All wash steps were 6 washes with 200 µl of wash buffer and incubation steps 1 hour at room temperature unless stated otherwise. Plates were washed and incubated with 100 µl of blocking buffer

(PBS with 1 % BSA). Plates were washed just twice with wash buffer. A standard curve of recombinant bovine IL-10 (kindly provided by Mr Sean Wattegedera, Moredun Research Institute) was added to each plate, with all samples diluted 1 in 4 (50 µl per well). After incubation, wells were washed and then incubated with 50 µl of secondary antibody (mouse anti-bovine IL-10 biotinylated; clone CC320). Plates were washed and then incubated with 50 µl per well of streptavidin-HRP (P0397, Dako, Ely, UK) at room temperature for 45 minutes. Plates were washed and then developed with 100 µl per well of TMB substrate; the reaction was stopped with 50 µl per well of TMB stop solution.

4.2.9 Bovine IFN-γ ELISpot

A commercially available kit was used (ELISpot for Bovine IFN-γ; 3119-2HW-plus; MabTech, Nacka strand, Sweden) following the manufacturer's instructions. Briefly, PBMC were stimulated with complete culture media containing either PBS, OVA (10 µg/ml) or ConA (10 µg/ml) for 18 hours at 37 ^{o}C and 5% CO_2 before addition to hydrophobic PVDF membrane ELISpot plates (Merck Millipore, Nottingham, UK) in duplicate wells at either 2 $\times 10^5$ cells per well or 2 $\times 10^4$ cells per well. Following a further 24 hours incubation at 37 ^{o}C and 5 % CO_2, cells were washed off and spots developed by addition of ELISpot TMB substrate; spots per well were quantified automatically using an ELISpot reader (AID ELISpot reader version 4, Autoimmun Diagnostika Gmbh, Strasberg, Germany). To determine numbers of OVA-specific IFN-γ producing cells, the mean number of spots per well from unstimulated duplicate cultures were subtracted from those obtained from OVA-stimulated cultures. The results were expressed as spot forming units (SFU) per 1 $\times 10^6$ cells.

Table 10: Monoclonal antibodies used in ELISAs to detect cytokines and OVA specific antibodies.

Reactivity	Host/Clone	Isotype	Conjugate	Supplier	Dilution
Bovine IgA	Mouse/ K84.2F9	IgG_1	None	AbD Serotec (MCA628)	1 in 1000
Bovine IgG_1	Mouse/ K37.2G6	IgG_1	None	AbD Serotec (MCA627)	1 in 1000
Bovine IgG_2	Mouse/ K192.4F10	IgG_1	None	AbD Serotec (MCA626)	1 in 1000
Mouse IgG_1	Rat/ LO-MG1-2	IgG	HRP	AbD Serotec (MCA336P)	1 in 1000
Bovine IL-10	Mouse/ CC318	IgG_{2b}	None	BioRad (MCA2110)	4 µg/ml
Bovine IL-10	Mouse/ CC320b	IgG	Biotin	BioRad (MCA2111B)	1 µg/ml
Bovine IFN-γ	Mouse/ MT17.1	IgG_1	None	MabTech (3119-3)	2 µg/ml
Bovine IFN-γ	Mouse/ MT307b	IgG_{2a}	Biotin	MabTech (3119-6)	1 in 2,000

4.2.10 Flow cytometry to detect OVA-specific $CD25^+CD4^+$ and $CD25^+CD8^+$ T-cells in PsLN

For flow cytometric analysis of leukocyte population, PsLN cells were seeded at 2×10^5 cells per well into 96 well round bottom plates. Triplicate wells for each animal were stimulated with OVA (10 μg/ml), Con A (10 μg/ml [Sigma-Aldrich]) or an equivalent volume of PBS (unstimulated control) in RPMI 1640 (Invitrogen, Paisley, UK) containing 10 % Hi-FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-mercaptoethanol. Plates were incubated for 5 days at 37 ºC at 5 % CO_2. Cell were harvested and antibody labelling was performed in 96-well round bottomed plates as follows: cells were washed once with flow cytometry buffer (PBS + 5 % Hi FCS + 0.02 % sodium azide) before incubation for 30 minutes at 4 ºC with the following pairs of monoclonal antibodies: anti-bovine CD4 plus anti-bovine CD25 or anti-bovine CD8 plus anti-bovine CD25. Additional cells were also incubated in parallel with each antibody separately to act as Fluorescence Minus One (FMO) controls, or with appropriate isotype controls. Cells were pelleted by centrifugation at 1,200 rpm for 1 minute and washed twice with flow cytometry buffer. Cells were then incubated with both anti-mouse IgG_1 conjugated to Alexa Fluor® 647 and anti-mouse IgG_{2a} conjugated to Alexa Fluor® 488 for 30 minutes at 4 ºC. Cells were pelleted and washed twice with flow cytometry buffer and finally re-suspended in PBS. Immediately prior to analysis, SYTOX™ Blue Nucleic acid stain (ThermoFisher Scientific, Loughborough, UK) was added to cells to discriminate live and dead cells. Details of all antibodies used are shown in Table 11.

Table 11: Antibodies used in flow cytometry to detect cellular responses.

Reactivity	Clone/ Host	Isotype	Conjugate	Supplier	Dilution
Bovine CD8	IL-A105	IgG_{2a}	None	Tim Connelley[215]	1:4000
Bovine CD4	Il-A12	IgG_{2a}	None	Tim Connelley[216]	1:4
Bovine CD25	IL-A111	IgG_1	None	AbD Serotec	1:200
Mouse IgG_1	Goat	N/A	AlexaFlour®647	AbD Serotec	1:2000
Mouse IgG_{2a}	Goat	N/A	AlexaFlour®488	AbD Serotec	1:2000
Isotype control	Mouse	IgG_1	None	AbD Serotec	1:50
Isotype control	Mouse	IgG_{2a}	None	AbD Serotec	1:50

Cells were analysed on a MACS Quant flow cytometer (Miltenyi Biotec, Surrey, UK) and the data was analysed using Flow Jo software version 10 (FlowJo, Ashland, USA). Figure 33 panel A demonstrates lymphocyte gating by side and forward scatter, doublets were discriminated as in Figure 33 panel B and dead cells were discriminated by fluorescence in the VioBlue channel by SYTOX™ Blue staining as in Figure 33, panel C. Gating for antibody labelling of bovine CD4, CD8 and CD25 was set using fluorescence minus one (FMO) stained cells as detailed in Figure 34. OVA-specific activation was expressed as the fold change in the percentage of $CD4^+CD25^+$ and $CD8^+CD25^+$ cells in OVA-stimulated cells compared to unstimulated controls.

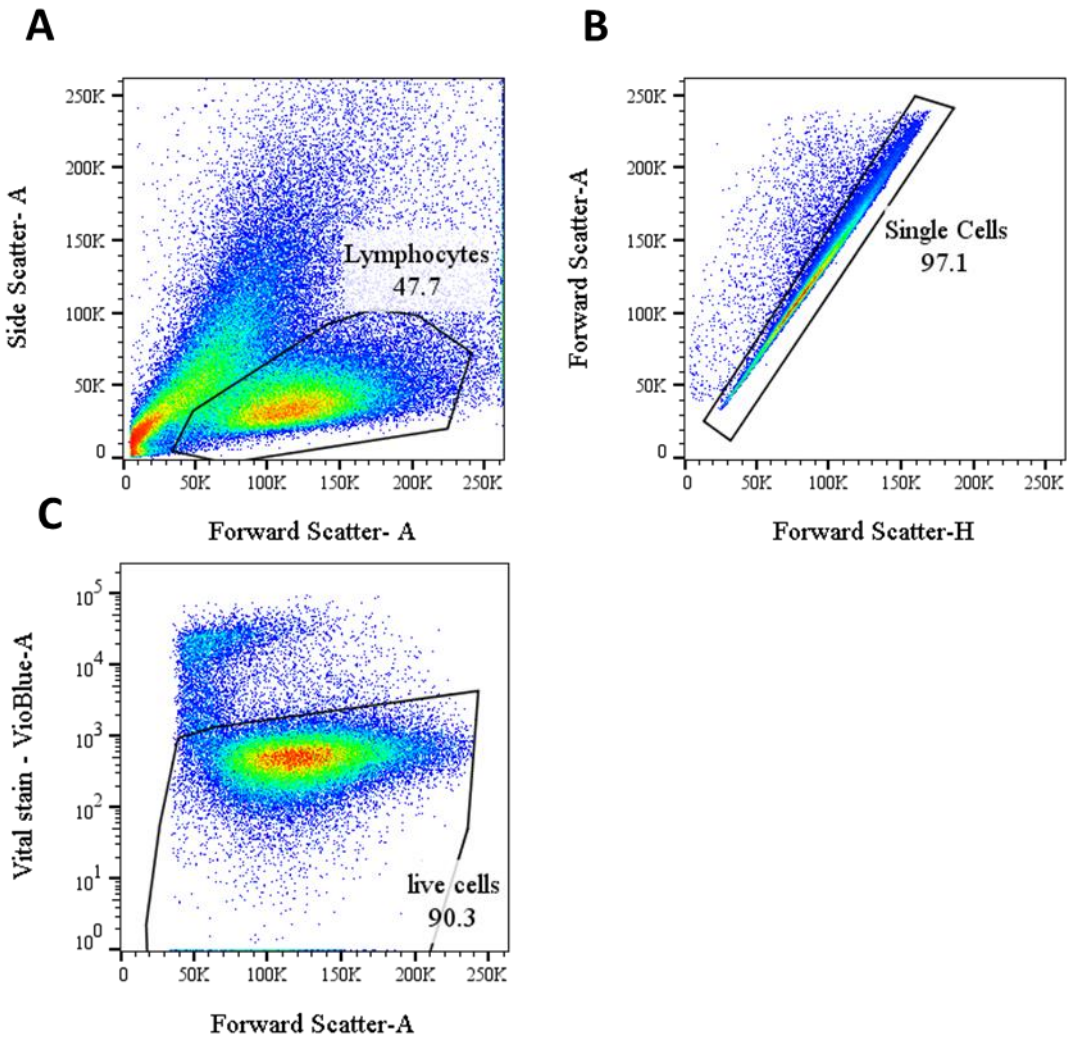


Figure 33: The gating strategy used to determine single live lymphocytes for the quantitation of activated (i.e. $CD25^+$) $CD4^+$ and $CD8^+$ pre-scapular lymph node cells by flow cytometry. A, is pulse area and H, is maximum pulse amplitude (height). Numbers represent the percentage of events in each gate. Figure A is all the events captured by the flow cytometer, the gate is lymphocytes captures, B is the events sitting in the lymphocyte gate in panel A and the gate captures single lymphocytes and allows discrimination against doublets; and C is the events sitting in the single cells gate in panel B, the gate represents live cells discriminated versus dead cells by SYTOX™ Blue staining, fluorescence in the VioBlue A channel.

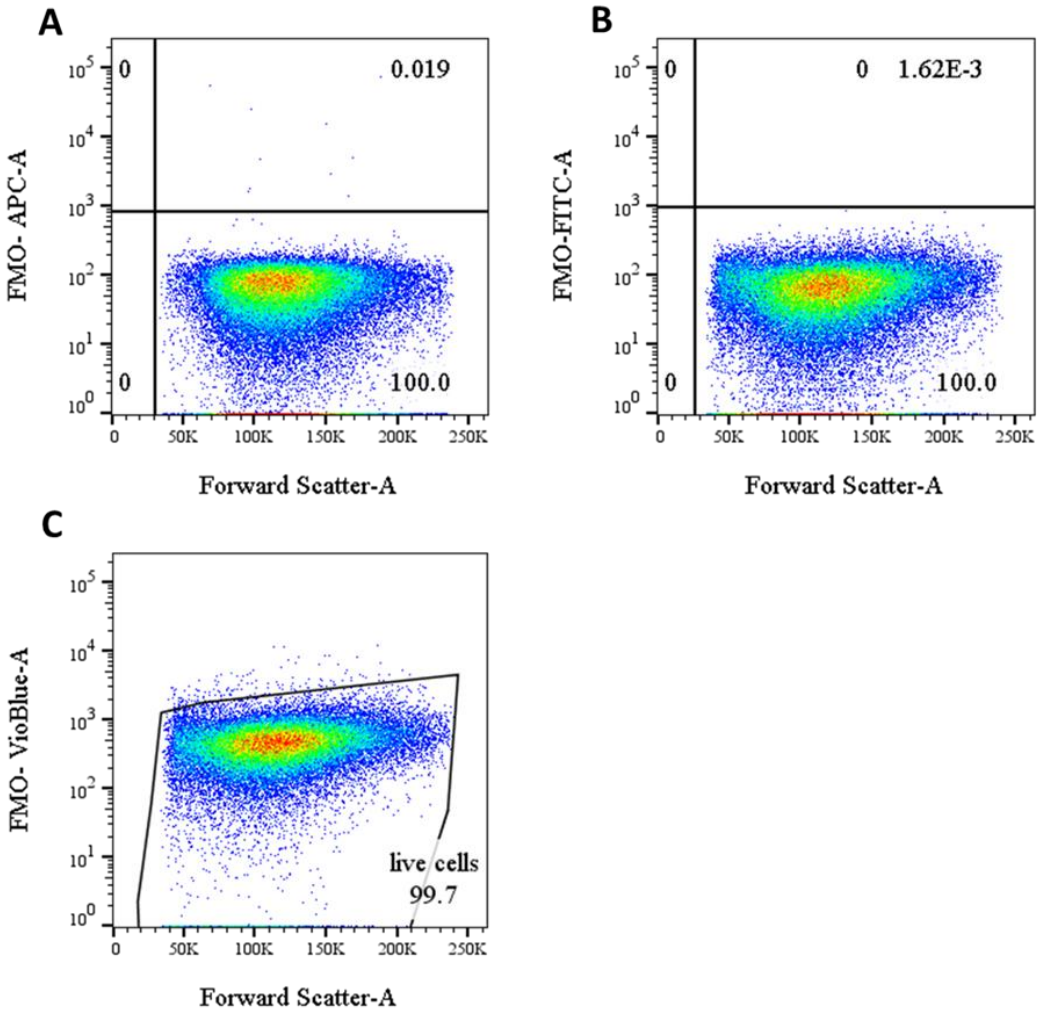


Figure 34: Fluorescence minus one controls of stained pre-scapular lymph node cells. A is pulse area. Events are pre-gated for lymphocytes, singles and live cells (except C). Numbers represent the percentage of events in each gate. Cytometer channels are FITC = 488 nm laser, APC = 635 nm laser and VioBlue = 405 nm laser.

4.2.11 Statistical analyses

Mixed model statistical analysis and the combined analysis of Trial 1 and Trial 2 data was performed using R version 10 by Dr Javier Palarea (Biomathematics and Statistics Scotland; BioSS). For each STEC O157 strain (PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+) the data was analysed separately, comparing OVA immune responses from challenged and immunised animals (C-I) with the appropriate unchallenged and immunised (I-only) or challenged only animals (C-only). The OVA-specific lymphocyte stimulation index data from PBMC was analysed by log transforming the OVA-specific stimulation index and then a generalised additive model (GAM) with identity link function and Gaussian errors was fitted by restricted maximum likelihood (REML) to allow investigation of the effect of treatment (STEC challenge and/ or OVA-immunisation) on stimulation index over the duration of the experiment. It included treatment as fixed effect and spline-based smooth terms (one per treatment) to fit the non-linear relationships of the response with time. Heterogeneous variances by treatment were allowed. Pair-wise linear hypotheses tests were subsequently used to compare between treatments and FDR corrections.

ELISpot (OVA specific IFN-γ releasing cells) data was analysed by log transformation of the OVA-specific spot forming units per 10^6 +1. A random intercept mixed model including treatment, time and their interaction as fixed effect and animal as random effect was fitted using REML. An exponential within group variance structure over time was considered to model non-homogenous variances between treatment groups. Again pair-wise linear hypotheses tests were subsequently used to compare between treatments and FDR corrections.

A GAM with identity link function and Gaussian errors was fitted by REML to investigate the effect of treatment on the OVA-specific IgG_1 and IgG_2 antibody levels in serum over time and the systemic OVA-specific IL-10 responses over time. It included treatment as fixed effect an spline-based smooth terms to fit the non-linear relationships of the response with time. Heterogeneous variances by group were allowed.

Data originating from PsLN cells and ASC probes were analysed with each trial using non-parametric tests using GraphPad prism (Version 7, La Jolla, USA). For Trial 1 where > 2 treatment groups existed a Kruskal-Wallis *H* test was used followed by Dunns multiple

comparisons tests if the overall *p*-value was < 0.05. For Trial 2 in which only two treatment groups existed, a Mann-Whitney *U* test was employed.

Finally, a combined analysis of some of the PsLN data from the first PT21/28 Stx2c+ trial and the second PT21/28 Stx2c+ trial was performed by Dr Javier Palarea using a two-way ANOVA approach of the log transformed response data to look for interactions between the repeats of the PT21/28 Stx2c+ trials. Treatment and trial were the main effects and the interaction between the two trials was analysed.

For all statistical tests a *p*- value of < 0.05 was considered significant.

4.3 Results

4.3.1 Trial 1- Bacterial shedding

Individual shedding curves for each calf orally challenged with STEC O157 are shown in Figure 35. All calves were shedding greater than 10^3 CFU/g faeces for the first three days post oral challenge and continued to shed for at least 7 days. These results confirm that the subsequent immunological analyses were conducted on successfully colonised calves and the calves were colonised with STEC O157 when the primary OVA immunisation was administered at day 5. All faecal samples from un-challenged control calves were negative for STEC O157 throughout the study (data not shown).

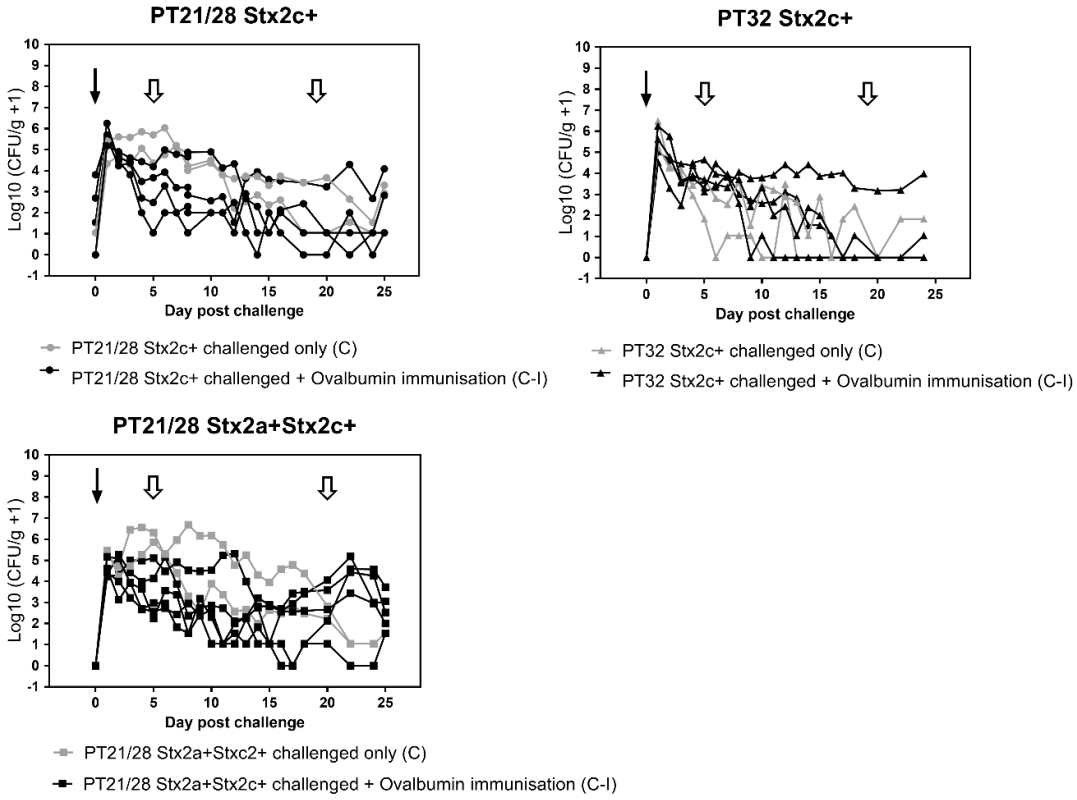


Figure 35: Faecal shedding curves of STEC O157 after experimental challenge of weaned calves. Six or seven calves in each trial were orally challenged once with ~10^9 CFU of STEC O157 (PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+ Stx2c+) indicated by the black arrow. Calves were immunised with OVA plus Quil A via the subcutaneous route on two separate occasions indicated by the open arrows. For each strain, two calves received Quil A alone. The shedding data is expressed as log10 CFU/g faeces + 1. Each curve represents an individual animal. The grey symbols represent calves that were challenged only and the black symbols calves which were both OVA immunised and STEC O157 challenged.

4.3.2 Trial 1- Systemic OVA-specific cellular immune responses

Figure 36 demonstrates OVA-specific cellular immune responses in calves challenged with either PT21/28 Stx2c+, PT32 Stx2c+ or PT21/28 Stx2a+Stx2c+ STEC O157 strains. Overall for the PT21/28 Stx2c+ strain, there was a statistically significant difference in OVA-specific proliferation between the three treatment group ($p = 0.0023$); subsequent pairwise testing revealed statistically significant differences between challenged only (C-only) and immunised only (I-only) groups ($p = 0.0006$) but not between I-only and challenged and immunised (C-I) groups ($p = 0.1054$) or C-I and C-only ($p = 0.1873$). Overall for the PT32 Stx2c+ strain, there was a statistically significant difference in OVA-specific proliferation between the three treatment groups ($p < 0.0001$); subsequent pairwise testing revealed no statistically significant differences between the I-only and C-I groups ($p = 0.2468$), there was a statistically significant difference between the C-I and C-only groups ($p < 0.0001$) and also between the C-only and I-only groups ($p < 0.0001$). Overall for the PT21/28 Stx2a+Stx2c+ strain there was a statistically significant difference in OVA-specific proliferation between the three treatment groups ($p < 0.0001$); subsequent pairwise testing revealed no statistically significant differences between the I-only and C-I groups ($p = 0.0608$), there was a statistically significant difference between the C-I and C-only groups ($p < 0.0001$) and also between the C-only and I-only groups ($p < 0.0001$).

Overall for the PT21/28 Stx2c+, there was no statistically significant difference in OVA-specific IFN-γ spot forming units between the three treatment groups ($p = 0.4487$). Overall for the PT32 Stx2c+ strain, there was a statistically significant difference in OVA-specific IFN-γ sport forming units between the three treatment groups ($p = 0.0034$); subsequent pairwise testing revealed no significant differences between the I-only and C-I groups ($p = 0.2559$), there was a statistically significant difference between the C-I and C-only groups ($p = 0.0264$) and no statistically significant difference between the C-only and I-only groups ($p = 0.1089$). Overall for the PT21/28 Stx2a+Stx2c+ strain, there was a statistically significant differences in OVA-specific IFN-γ spot forming units between the three treatment groups ($p = 0.0171$); subsequent pairwise comparisons revealed no significant differences between the I-only and C-I groups ($p = 0.1476$), there was a statistically significant difference between the C-I and C-only groups ($p = 0.0193$) and also between the C-only and I-only groups ($p = 0.008$).

OVA-specific systemic IL-10 cytokine levels were determined by ELISAs in supernatants collected from OVA-stimulated PBMC from calves challenged with PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ STEC O157 strains. The results are shown in Figure 37. Overall for the PT21/28 Stx2c+ strain, there was a statistically significant difference in OVA-specific IL-10 between the three treatment group ($p = 0.0013$); subsequent pairwise testing revealed statistically significant differences between challenged only (C-only) and immunised only (I-only) groups ($p = 0.0004$) but not between I-only and challenged and immunised (C-I) groups. Overall for the PT32 Stx2c+ strain, there was no statistically significant difference in OVA-specific IL-10 between the three treatment groups ($p = 0.1080$). Overall for the PT21/28 Stx2a+Stx2c+ strain there was a statistically significant difference in OVA-specific IL-10 between the three treatment groups ($p < 0.0001$); subsequent pairwise testing revealed statistically significant differences between all three treatment groups, C-I versus C-only ($p = 0.0002$), C-I versus I-only ($p = 0.0231$) and also C-only versus I-only ($p < 0.0001$). The C-I group had generally lower OVA-specific IL-10 levels than the I-only group.

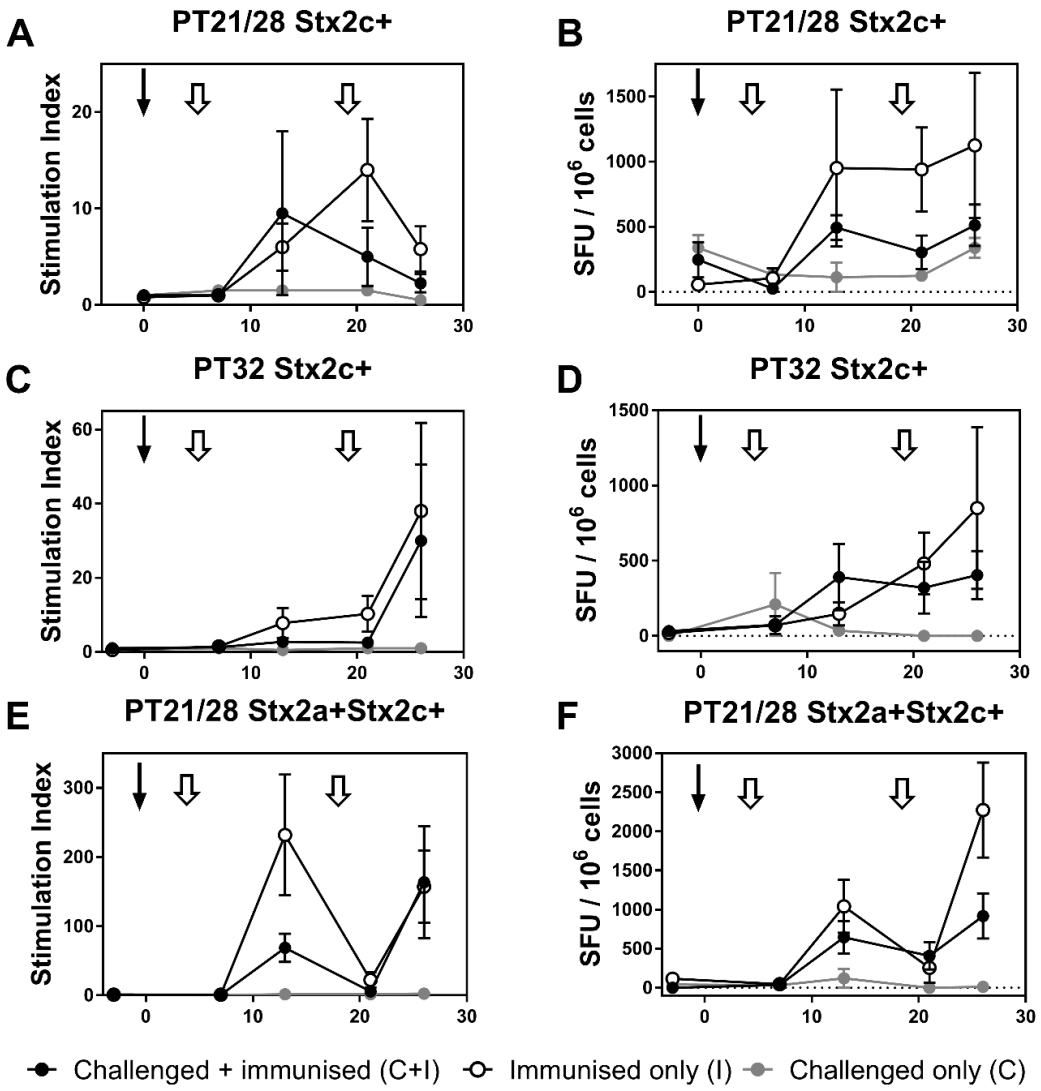


Figure 36: OVA-specific lymphocyte proliferation and bovine IFN-γ spot forming units in PBMC prepared at weekly time points throughout the PT21/28 Stx2c+ (A and B), PT32 Stx2c+ (C and D) and PT21/28 Stx2a+Stx2c+ (E and F) challenge experiments within trial 1. For each strain, calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). Proliferation (A, C and E) determined by a lymphocyte stimulation assay is expressed as stimulation indices, representing fold changes in the response to OVA from levels with the relevant PBS control. Bovine IFN-γ releasing cells were determined by bovine IFN-γ ELISpot (B, D and F) and expressed as spot forming units per 10^6 cells in response to OVA, responses of non-stimulated (PBS) cultures were subtracted before the data was analysed. The circles represent the mean and errors bars the standard error of the mean (SEM). Black arrows represent STEC O157 challenge and open arrows OVA immunisations.

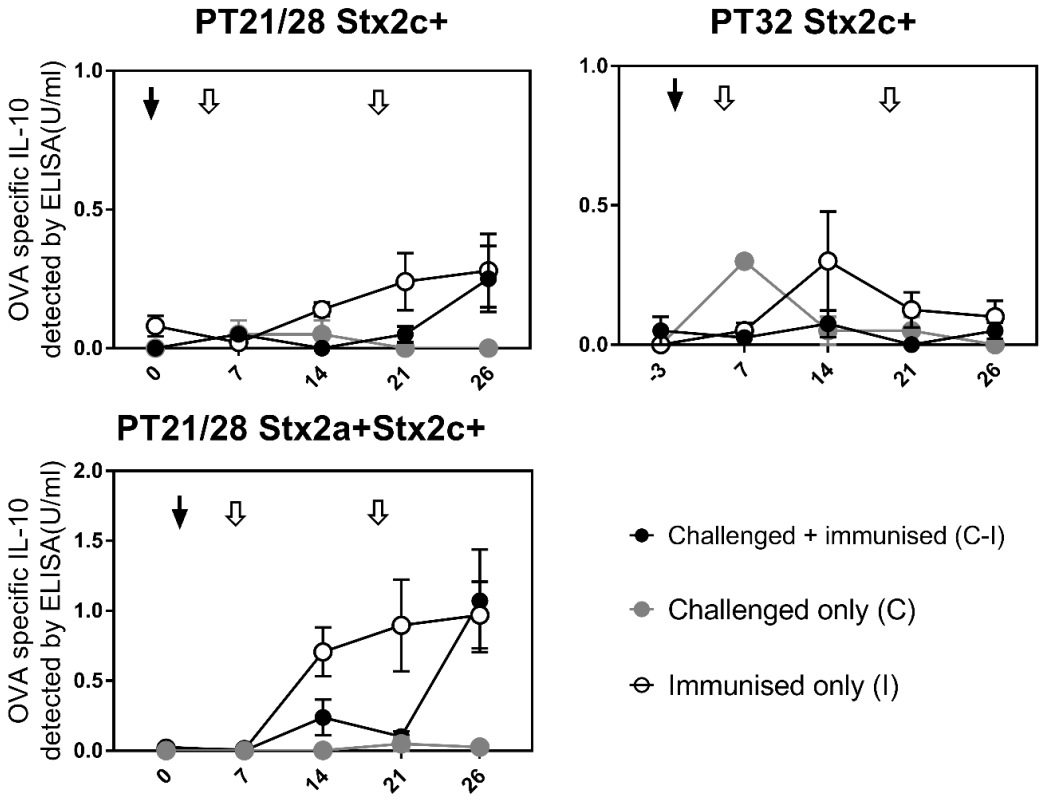


Figure 37: OVA-specific IL-10 release from PBMC prepared at weekly time points throughout the PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ challenge experiments within trial 1. For each strain calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). PBMC were stimulated with OVA or PBS for 4 days and levels of OVA-specific IL-10 release determined at day 5 by ELISA, OVA-specific response were calculated by subtracting background IL-10 amounts (PBS stimulated) from OVA stimulated levels. The black arrows represent STEC O157 challenge and the open arrows OVA immunisations. Black symbols represent challenged and OVA immunised calves (C-I), black open symbols calves that were only OVA immunised (I) and grey symbols calves that were STEC O157 challenged but not OVA immunised (C). The symbols represent the mean for each group and the error bars the standard error of the mean (SEM).

4.3.3 Trial 1- OVA-specific systemic antibody responses

Figure 38 compares systemic OVA-specific IgG_1 and IgG_2 antibody responses in calves challenged with PT21/28 Stx2c+, PT32 St2xc+ and PT21/28 Stx2a+Stx2c+ STEC O157 strains. Overall for the PT21/28 Stx2c+ strain, there was a statistically significant difference in mean OVA-specific IgG_1 responses between the three treatment groups ($p < 0.0001$); subsequent pairwise analysis revealed significant differences between I-only and C-I groups ($p = 0.0008$), C-I and I-only groups ($p < 0.0001$) and also C-only and I-only groups ($p = 0.0228$). Overall for the PT21/28 Stx2c+ strain, there was a statistically significant difference in mean OVA-specific IgG_2 responses between the three treatment groups ($p = 0.0001$); but subsequent pairwise analysis did not reveal any statistically significant differences between the I-only and C-I groups ($p = 0.1692$), there was a statistically significant difference between the C-I and C-only groups ($p = 0.0003$) and also between the C-only and I-only groups ($p = 0.112$).

Overall for the PT32 Stx2c+ strain, there was no statistically significant difference in mean OVA-specific systemic IgG_1 ($p = 0.2810$) or IgG_2 ($p = 0.1180$) between the three treatment groups. Overall for the PT21/28 Stx2a+Stx2c+ strain there was a statistically significant difference in mean OVA-specific systemic IgG_1 ($p < 0.001$) between the three treatment groups; subsequent pairwise comparisons did not reveal any statistically significant differences between the I-only and C-I groups ($p = 0.6290$), there was a statistically significant difference between the C-I and C-only groups ($p = 0.0014$) and also between the C-only and I-only groups ($p < 0.0001$). Overall for the PT21/28 Stx2a+Stx2c+ strain there was no statistically significant difference in mean OVA-specific systemic IgG_2 ($p = 0.5010$) between the three treatment groups.

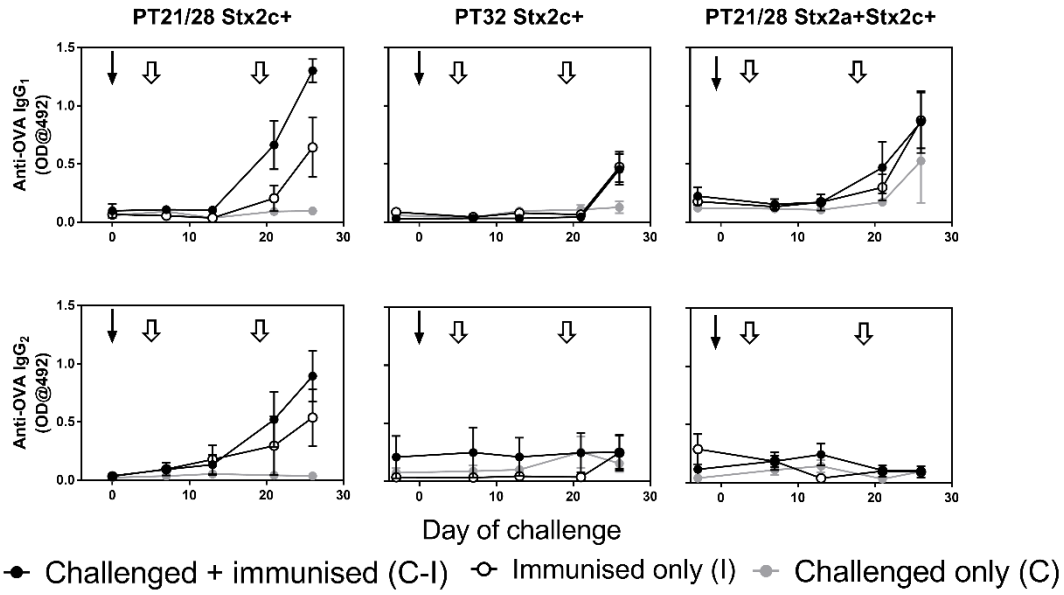


Figure 38: OVA-specific antibody levels in serum prepared at weekly time points throughout the PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+ challenge experiments within trial 1. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). The OVA specific antibody levels were determined by ELISA for each isotype (IgG_1 or IgG_2) and are expressed as the mean optical density (OD) at 492 nm of duplicate wells normalised to a positive control sample. The symbols represent the mean for each group and error bars the SEM. Black arrows represent the oral STEC O157 challenge and open arrows OVA immunisations.

4.3.4 Trial 1- PsLN OVA-specific immune responses

Figure 39 indicates OVA-specific proliferation in cells from the PsLN. There was an overall statistically significant difference between the three treatment groups for the PT21/28 Stx2c+ trial OVA-specific lymphocyte proliferation (p = 0.0464), with mean proliferation being highest in the C-I group and similar between I-only and C-only groups; there was no statistically significant difference between the C-I and I-only group following pairwise comparisons. The PT32 Stx2c+ calves also followed the same trends but there were no statistically significant differences (p = 0.3921) between the three treatment groups. In the PT21/28 Stx2a+Stx2c+ experiment the three treatment groups showed no statistically significant differences in their OVA-specific lymphocyte simulation response (p = 0.0961).

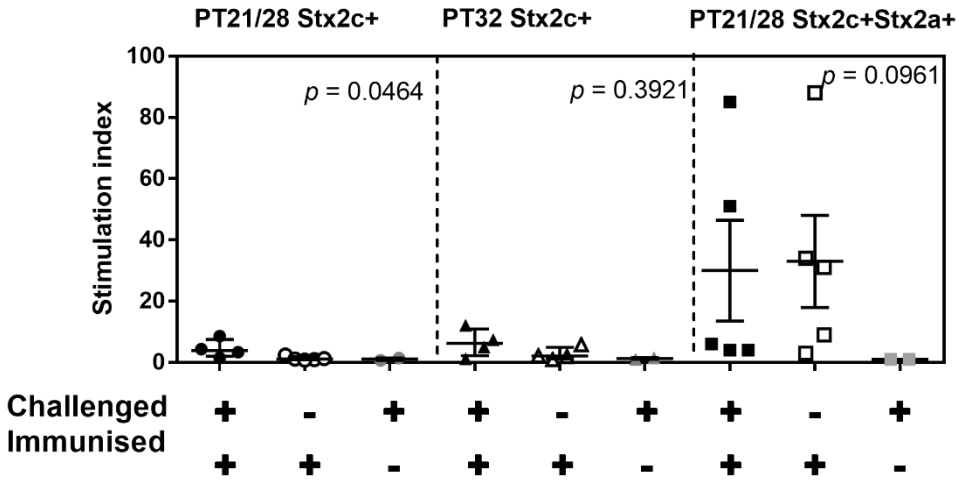


Figure 39: OVA-specific proliferation in bovine lymph node cells isolated post mortem from the PsLN. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). Proliferation was determined by a lymphocyte stimulation assay and is expressed as stimulation index, representing changes in the response to OVA from levels with the relevant PBS control. Symbols represent individual animals within the experiments within trial 1, black symbols are challenged and immunised calves (C-I), clear symbols OVA immunised only (I) and grey symbols challenged only calves (C). Error bars represent median and IQR of the group. The p-values were determined by Kruskal-Wallis H test for differences between the treatments (for each strain) and are expressed as overall p-values.

Figurse 40 and 41 shows the gating strategy for flow cytometry used to determine activated ($CD25^+$) $CD4^+$ and $CD8^+$ T-cells in the PsLN cells following *ex vivo* stimulation with OVA. Figure 42 shows OVA-specific $CD4^+CD25^+$ and $CD8^+CD25^+$ cells in the PsLN. Overall for the PT21/28 Stx2c+ experiments, there was a statistically significant difference between the three treatment groups in the number of OVA-specific PsLN $CD8^+CD25^+$ cells ($p = 0.0364$); subsequent pairwise comparisons showed no significant differences between I-only and C-I groups, however the trend was for a more OVA-specific $CD8^+CD25^+$ cells in the C-I group compared to the I-only group. Overall for the PT32 Stx2c+ strain, there was no statistically significant differences between the three treatment groups in the OVA-specific pre-scapular $CD8^+CD25^+$ cells ($p = 0.1482$). Overall for the PT21/28 Stx2a+Stx2c+ strain, there was no statistically significant differences between the three treatment groups in the OVA-specific pre-scapular $CD8^+CD25^+$ cells ($p = 0.0824$).

There were no statistically significant differences between the OVA-specific pre-scapular $CD4^+CD25^+$ cells, in either the PT21/28 Stx2c+ ($p = 0.0641$), PT32 Stx2c+ ($p = 0.0628$) or the PT21/28 Stx2a+Stx2c+ strains ($p = 0.9623$) between the three treatment groups.

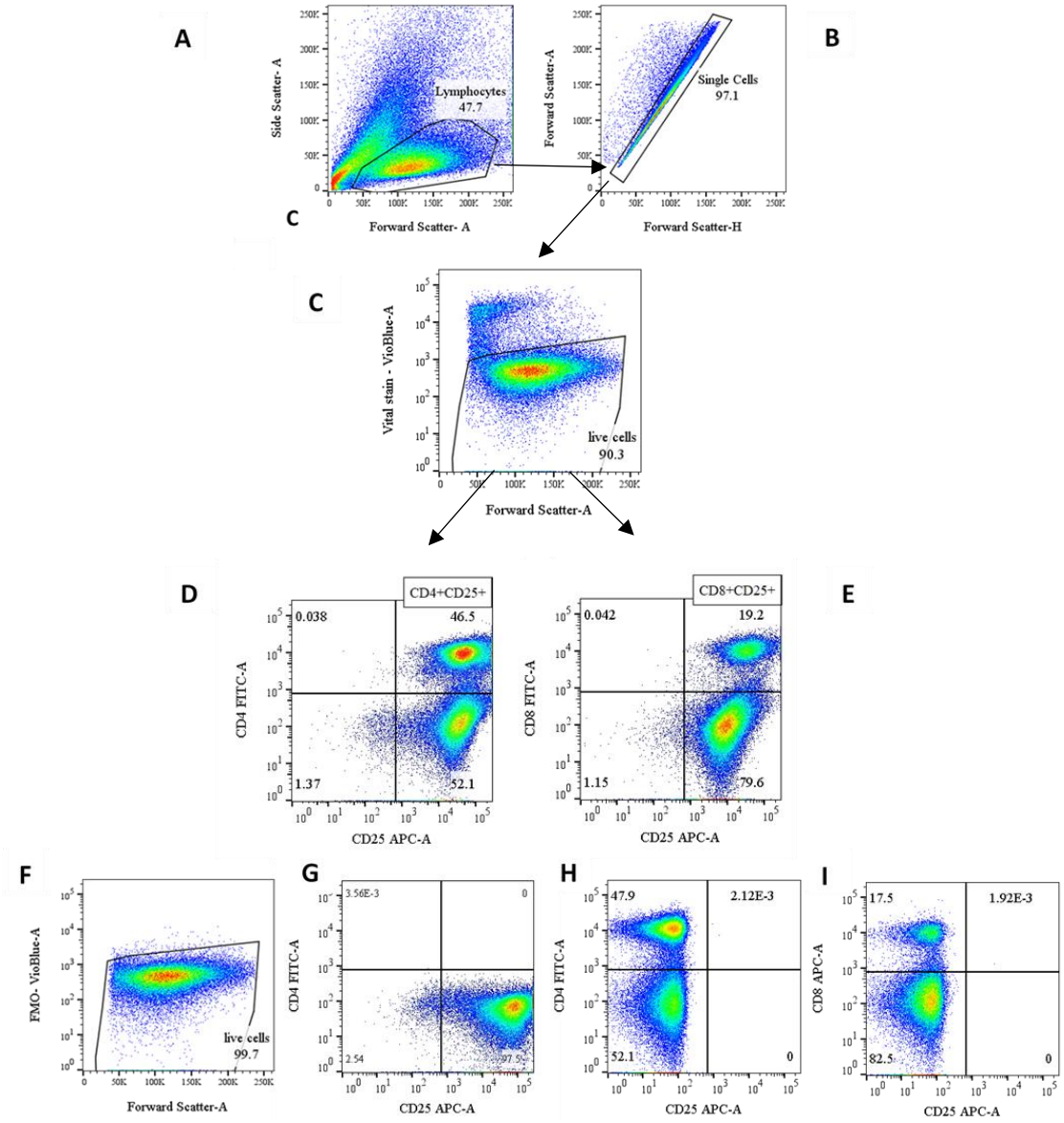


Figure 40: Gating strategy used to determine single live activated (i.e. $CD25^+$) $CD4^+$ or $CD8^+$ pre-scapular lymph node cells by flow cytometry. Figure A is all the events captured, the gate is lymphocytes, figure B is lymphocytes captured and the gate captures single lymphocytes. Figure C is the events sitting in the single cell gate and the gate represents live cells discriminated versus dead cells by SYTOX™ Blue staining (fluorescence in the VioBlue channel). Figures F, G, H and I are FMO controls. Figure F is SYTOX Blue FMO. Figures D, E, G, H and I capture events gated for single, live lymphocytes, they are cells stimulated with ConA and subsequently labelled for surface expression of CD25 (with APC) and either CD4 or CD8 (FITC). Figure G is FMO for CD4 and CD8, H is FMO for CD25 (with CD4 labelling) and I is FMO for CD25 (with CD8 labelling). The quadrant gating represents the gating for $CD4^+$/CD8+ and $CD25^+$

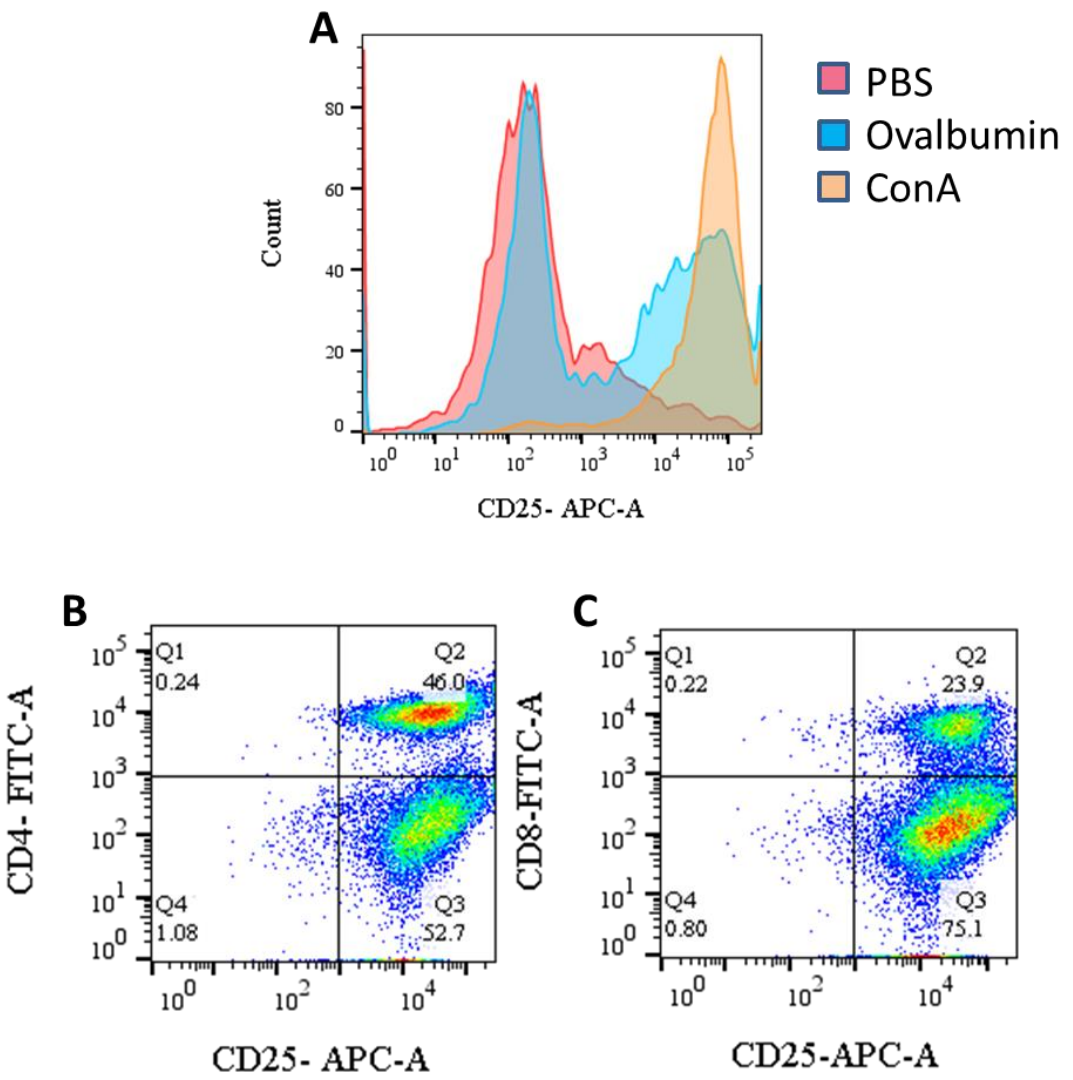


Figure 41: Representative flow cytometry histograms and density dot plots of CD25 expression on PsLN cells stimulated *ex vivo* for 5 days with Ovalbumin (OVA), Concanavalin A (ConA) or PBS. (A) histogram of cells from a single animal that have been pre-gated for lymphocytes, single and live cells, stimulated with PBS, ConA or OVA and subsequently labelled for surface expression of CD25 (with APC). (B) Dot plot of cells stimulated with ConA and subsequently labelled for surface expression of CD25 (with APC) and CD4 (with FITC). (C) Dot plot of cells stimulated with ConA and subsequently labelled for surface expression of CD25 (with APC) and CD8 (with FITC). Flow cytometer channels are FITC = 488 nm and APC = 635 nm.

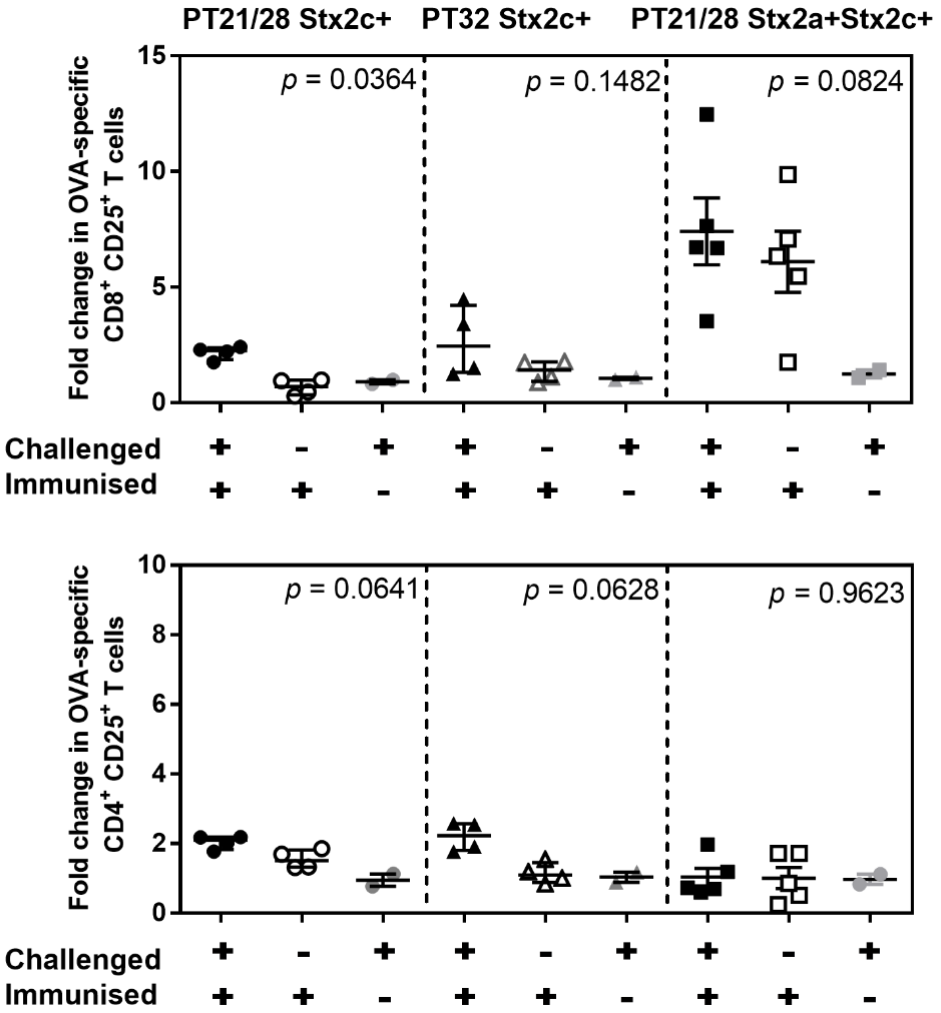


Figure 42: Levels of OVA-specific activated $CD4^+$ and $CD8^+$ T-cells in re-stimulated bovine PsLN cells isolated at post mortem. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). Cells were stimulated for 5 days with OVA or PBS and the activation status of $CD4^+$ and $CD8^+$ cells evaluated by surface expression of CD25, as determined by flow cytometry. OVA-specific activation as expressed as a fold change in double positive cells in OVA-stimulated cells compared to PBS stimulated controls. Symbols represent individual animals within the trial, black symbols are challenged and immunised calves (C-I), clear symbols OVA immunised only (I) and grey symbols challenged only calves (C). Error bars represent median and IQR of the group. The p-values were determined by Kruskal-Wallis H test for differences between the treatments (for each strain) and are expressed as overall *p*-values.

The OVA specific cytokine release from *ex vivo* stimulated PsLN cells was determined (Figure 43). Overall for the PT21/28 Stx2c+ strain, there was a statistically significant difference in OVA-specific IFN-γ release from stimulated PsLN cells between the three treatment groups (p = 0.0208); subsequent pairwise testing showed there was a statistically significant difference between the C-I and the I-only group (p = 0.0355) with more OVA specific IFN-γ released in the C-I compared to the I-only group. Overall for the PT32 Stx2c+ strain, there was no statistically significant difference in OVA-specific IFN-γ release from stimulated PsLN cells between the three treatment groups (p = 0.1042). Overall for the PT21/28 Stx2a+Stx2c+ strain, there was no statistically significant difference in OVA-specific IFN-γ release from stimulated PsLN cells between the three treatment groups (p = 0.1463). There were no statistically significant differences in OVA-specific IL-10 released from stimulated PsLN cells between the three treatment groups in either the PT21/28 Stx2c+ (p = 0.6277), PT32 Stx2c+ (p = 0.8533) or the PT21/28 Stx2a+Stx2c+ strain (p = 0.0545).

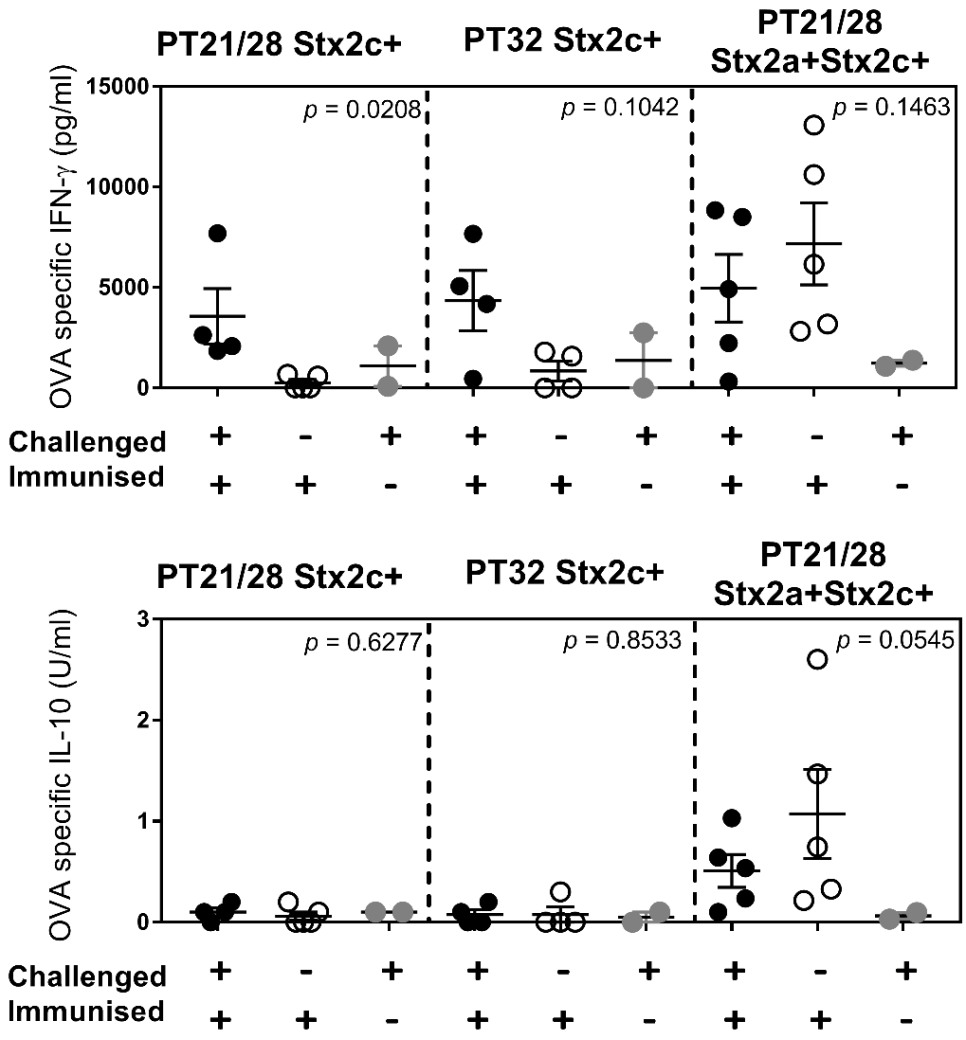


Figure 43: OVA-specific releases of bovine cytokines (IL-10 and IFN-γ) were determined by ELISA in supernatants from PsLN node cells stimulated for 5 days *ex vivo* with OVA. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). Symbols represent individual animals within the trial, black symbols are challenged and immunised calves (C-I), clear symbols OVA immunised only (I) and grey symbols challenged only calves (C). Error bars represent median and IQR of the group. The p-values were determined by Kruskal-Wallis H test for differences between the treatments (for each strain) and are expressed as overall *p*-values.

OVA-specific antibody levels were determined from antibody secreting cell probes generated from the PsLN cells (Figure 44). There were no statistically significant differences in OVA-IgA, OVA-IgG_1, or OVA-IgG_2 between C-I, I-only or C-only treatment for any of the three STEC O157 strains, although for the PT21/28 Stx2c+ strain, OVA-IgG_1 responses followed the same trend as seen with the serum antibody responses, being higher in the C-I group compared to the I-only control group ($p = 0.0505$).

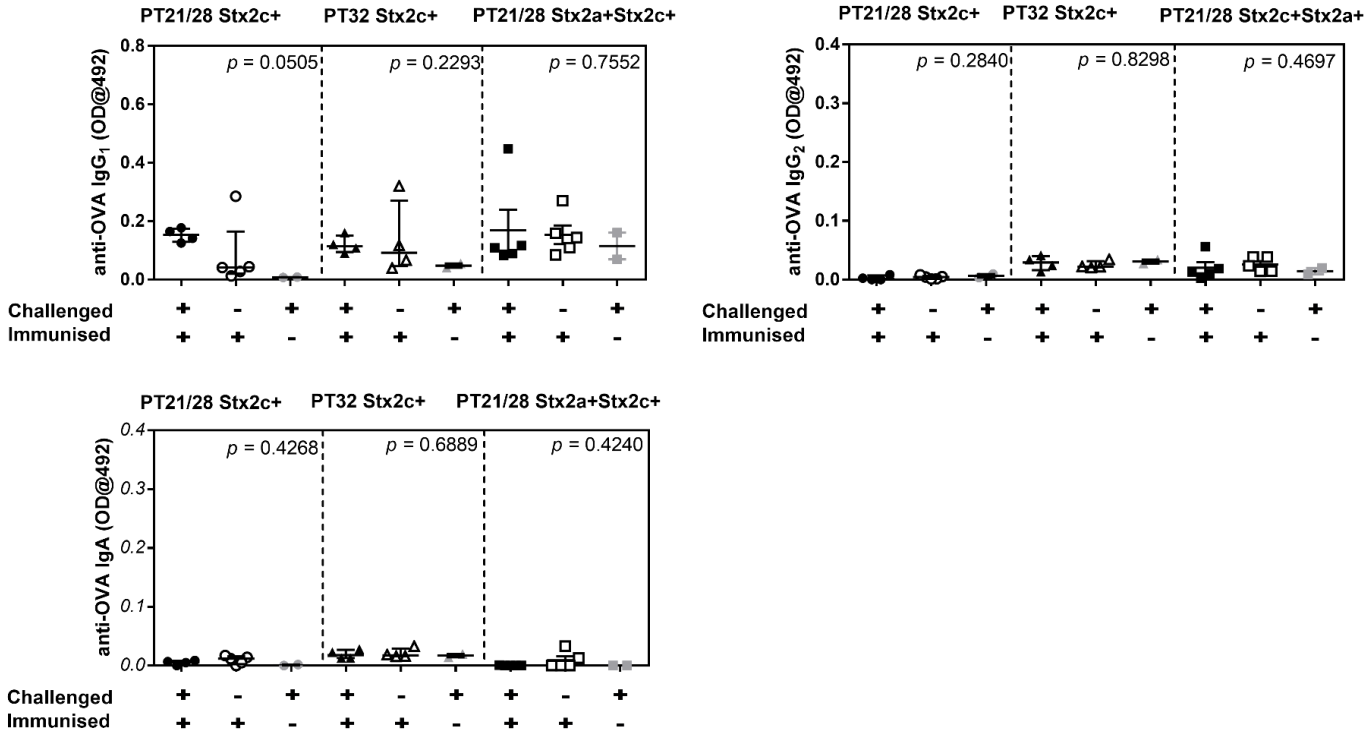


Figure 44: OVA-specific IgA, IgG_1 and IgG_2 antibody levels in antibody secreting cell probes generated from PsLN cells. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised), STEC O157 challenged but not immunised (Challenged only) or unchallenged but OVA immunised (Immunised only). Antibody levels determined by ELISA are expressed as the mean OD at 492 nm of duplicate wells normalised to a positive control sample. Symbols represent individual animals within the trial, black symbols are challenged and immunised calves (C-I), clear symbols OVA immunised only (I) and grey symbols challenged only calves (C). Error bars represent median and IQR of the group. The *p*-values were determined by Kruskal-Wallis H test for differences between the treatments within each strain.

4.3.5 Trial 2

The results from Trial 1 indicated a marginally statistically significant difference between the three treatment groups (C-I, I-only and C-only) in OVA-specific lymphocyte proliferation, OVA-specific IFN-γ release, OVA-specific $CD8^+CD25^+$ cells and anti-OVA IgG_1 in the PsLN cells in the PT21/28 Stx2c+ challenge. With a trend for increase in these OVA-specific responses in the C-I treatment group compared to both the I-only and C-only group with the PT21/28 Stxc2+ challenge. This suggested that colonisation with the PT21/28 Stx2c+ resulted in enhancement of OVA-specific immune responses in the PsLN. To validate these results, the trial was repeated.

Daily bacterial shedding by calves in the second PT21/28 Stx2c+ trial is shown in Figure 45. These results confirmed that the subsequent immunological analyses were conducted on successfully colonised calves and the calves were colonised when the primary OVA immunisation was administered at day 5. All faecal samples from un-challenged control animals were negative for STEC O157 throughout the trial (data not shown).

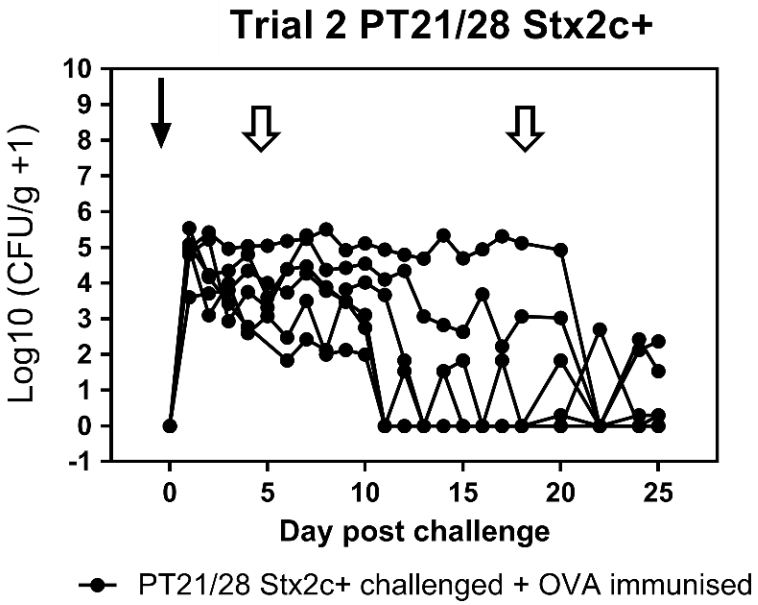


Figure 45: Faecal shedding curves of STEC O157 after experimental infection of weaned calves in the second PT21/28 Stx2c+ trial. Six calves were orally challenged with STEC O157 PT21/28 Stx2c+ indicated by the black arrow. The calves were also OVA immunised by subcutaneous injection on two separate occasions indicated by the open arrows. The shedding data is expressed as log_{10} CFU/g faeces+ 1. Each curve represents an individual animal.

4.3.6 Trial 2- Systemic OVA-specific immune responses

Figure 46 shows systemic OVA-specific IFN-γ release and lymphocyte proliferation of PBMC, and serum levels of OVA-specific IgG_1 and IgG_2 antibody responses in calves from PT21/28 Stx2c+ Trial 2. There was no statistically significant difference between treatments in the OVA-specific bovine IFN-γ (p = 0.0682, Figure 46 A). There was also no significant difference in the mean log (stimulation index) between the treatment groups (p = 0.0982, Figure 46 B). However, these results showed a similar trend to PT21/28 Stx2c+ calves challenged with Trial 1 with the I-only group having a higher (but not significantly) OVA-specific response than the C-I group.

Serum OVA-specific circulating IgG_1 antibody responses PT21/28 Stx2c+ are shown in Figure 46 C. A statistically significant difference between the C-I and the I-only group (p < 0.0006) was detected, with OVA-specific IgG_1 being higher in the I-only group. No

statistically significant differences in serum OVA-specific IgG_2 antibody levels were detected between the C-I or I only groups (p = 0.544, Figure 46 D).

OVA specific IL-10 cytokine levels in supernatants collected from OVA stimulated PBMC from calves challenged with PT21/28 Stx2c+ in the second trial did not statistically significantly differ between treatments (p = 0.18; Figure 47).

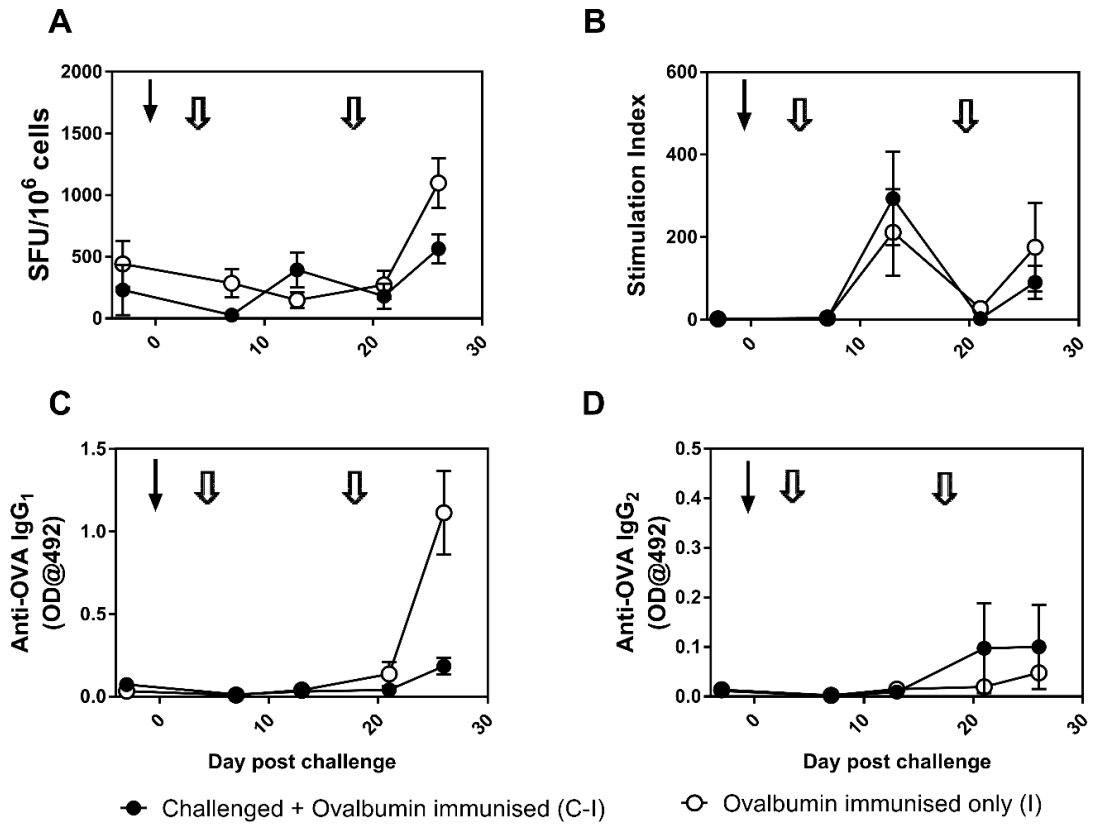


Figure 46: OVA-specific lymphocyte proliferation, bovine IFN-γ spot forming units in PBMC and IgG_1 and IgG_2 levels in serum prepared at weekly time points throughout the second PT21/28 Stx2c+ trial. Six calves were orally challenged with PT21/28 Stx2c+strain and OVA immunised (C-I) and six calves were OVA immunised only (I). Bovine IFN-γ positive cells were determined by ELISpot are expressed as spot forming units per 10^6 cells in response to OVA with the relevant background PBS controls subtracted (A); the circles represent the mean and error bars the SEM. OVA-specific proliferation (B) determined by a lymphocyte stimulation assay is expressed as indices, representing changes in response to OVA compared to non-stimulated cells. The OVA-specific IgG_1 (C) and IgG_2 (D) were determined by ELISA and are expressed as the mean OD of duplicate wells normalised to a positive control sample. The circles represent the mean of the groups and the error bars the standard error of the mean (C and D). Black arrows represent oral STEC O157 challenge and open arrows OVA immunisations.

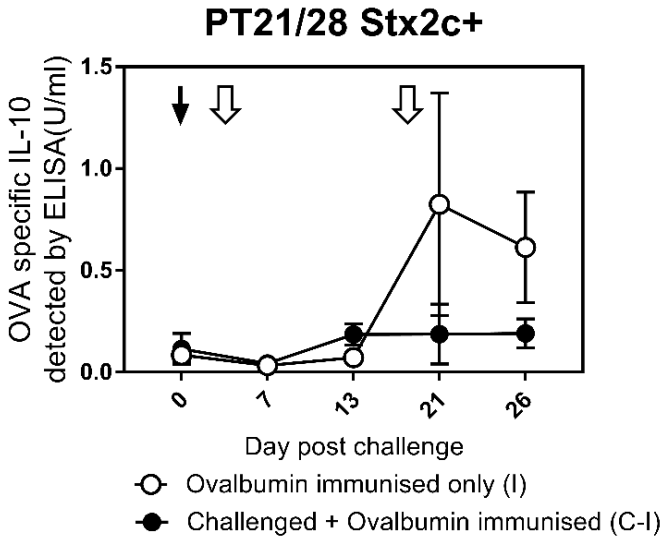


Figure 47: OVA-specific IL-10 release from PBMC prepared at weekly time points throughout the PT21/28 Stx2c+ second challenge trial. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised) or unchallenged but OVA immunised (Immunised only). PBMC were stimulated with OVA or PBS for 4 days and levels of OVA-specific IL-10 release determined at day 5 by ELISA. OVA-specific responses were calculated by subtracting background IL-10 amounts (PBS incubated) from OVA stimulated levels. The black arrows represent STEC O157 challenge and the open arrows OVA immunisations. Black symbols represent challenged and OVA immunised calves (C-I) and black open symbols calves that were only OVA immunised (I). The symbols represent the mean for each group and the error bars the standard error of the mean (SEM).

4.3.7 Trial 2- OVA-specific immune responses within PsLN

PsLN cells were prepared from calves in the second PT21/28 Stx2c+ trial at post mortem to allow analysis of the local immune response to OVA immunisations (Figure 48). OVA-specific lymphocyte proliferation of PsLN cells significantly differed between the two treatment groups with the C-I group showing higher OVA-specific lymphocyte proliferation than the I-only group (p = 0.0039, Figure 48 A). OVA-specific IgG_1 antibody levels within ASC probes generated from the PsLNs were also statistically significantly different between the two treatment groups with the I-only group having higher OVA-specific IgG_1 antibody levels than the C-I group (p = 0.0039, Figure 48 B).

Levels of OVA-specific $CD4^+CD25^+$ (activated $CD4^+$ T-cells) PsLN cells were not significantly different between the two treatment groups (C-I and I-only) (p = 0.07817, Figure 48 C). In contrast, OVA-specific $CD8^+$ $CD25^+$ PsLN cells were significantly different between the groups (p = 0.0374), being higher in the C-I group higher compared to the I-only group (Figure 48 D).

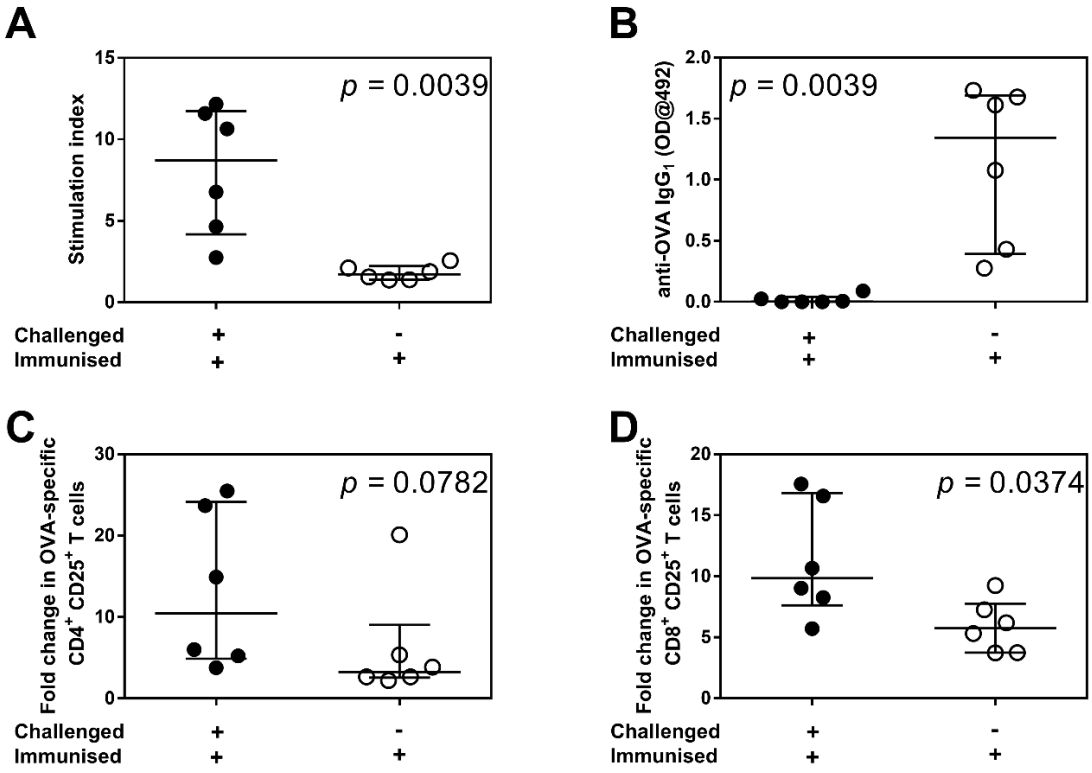


Figure 48: OVA-specific proliferation, IgG_1 levels, $CD8^+$ $CD25^+$ and $CD4^+CD25^+$ T cells of bovine PsLN cells isolated post-mortem from calves in the second PT21/28 Stx2c+ trial and stimulated *ex vivo* for 5 days with OVA. Six calves were orally challenged with PT21/28 Stx2c+ and OVA immunised (C-I) and 6 calves were OVA immunised only (I). Proliferation (A) determined by a lymphocyte stimulation assay is expressed as indices, representing fold changes in the response to OVA from levels with the relevant non-stimulated (PBS) control cultures. OVA-specific IgG_1 antibody levels (B) determined by ELISA are expressed as normalised optical density (OD) at 492 nm. OVA-specific $CD4^+CD25^+$ cells (C) and OVA specific $CD8^+CD25^+$ cells (D) determined by flow cytometry are expressed as fold changes in response to OVA from levels with relevant non-stimulated controls. Error bars represent median and IQR. The *p*-values were determined from by Mann-Whitney *U* (non-parametric) test for differences between the two groups.

The OVA specific cytokine release from *ex vivo* stimulated PsLN cells was determined (Figure 49). In Trial 2 with the PT21/28 Stx2c+ strain, there was a statistically significant difference in OVA-specific IL-10 release from stimulated PsLN cells between the two treatment groups ($p = 0.0022$) with the challenged calves having significantly lower levels of OVA-specific IL-10. There was no statistically significant difference in OVA-specific IFN-γ release between the two treatment groups ($p = 0.1797$).

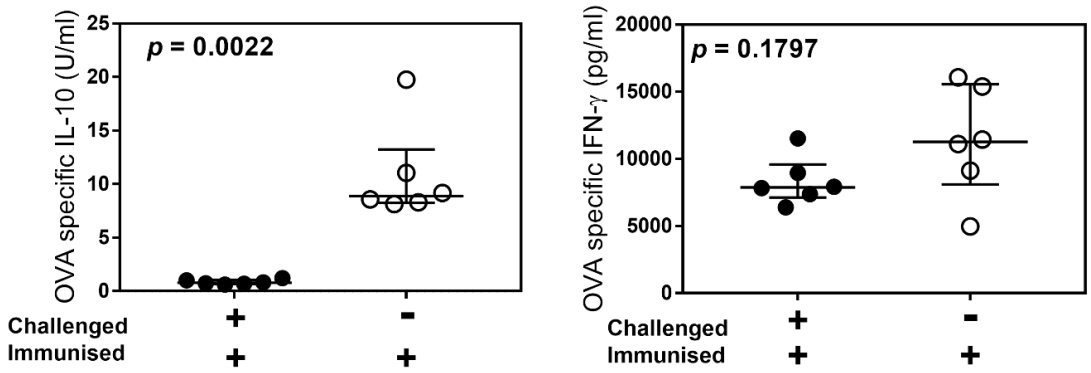


Figure 49: OVA-specific bovine cytokine releases (IL-10 and IFN-γ) were determined by ELISA in supernatant from PsLN node cells stimulated for 5 days *ex vivo* with OVA. Calves were STEC O157 challenged and OVA immunised (Challenged + immunised) or unchallenged but OVA immunised (Immunised only). Symbols represent individual animals within the trial, black symbols are challenged and immunised calves (C-I) and clear symbols OVA immunised only (I). Error bars represent median and IQR of the group. The *p*-values were determined by Mann-Whitney U test for differences between the two treatment groups.

4.3.8 Combined analysis of Trial 1 and Trial 2

In order to increase the statistical power of the analysis, some of the PsLN data relating to the PT21/28 Sx2c+ challenges in Trials 1 and 2 were combined and analysed together, with treatment and trial as main effects.

The combined OVA-specific proliferation data from the two PT21/28 Stx2c+ trials were analysed using a two-way ANOVA of the log transformed data and an interaction plot is shown in Figure 50. This analysis identified a statistically significant difference between treatment groups ($p < 0.0001$) with the C-I group showing higher mean OVA-specific

proliferation than the I-only in both trials. There was also a statistically significant difference in mean values between trials ($p = 0.037$), reflecting generally higher proliferative responses in Trial 2.

Combined OVA-specific IgG_1 data from ASC probes generated from both PT21/28 Stx2c+ trials were analysed together using a two-way ANOVA of the log transformed data and an interaction plot is shown in Figure 51. A statistically significant difference in mean OVA-specific IgG_1 in PsLN ASC probes was identified between the treatment groups ($p = 0.0005$); however a significant treatment × trial interaction was also identified ($p < 0.0001$). This reflected the different effects of colonisation with PT21/28 Stx2c+ strain on PsLN OVA-specific IgG_1 responses between the two trials, with OVA-specific IgG_1 response in the C-I group being significantly higher than those in the I-only group in Trial 1, but lower in Trial 2.

Finally, combined PsLN OVA-specific $CD4^+CD25^+$ and OVA-specific $CD8^+CD25^+$ cell data from both of the PT21/28 trials was analysed and interaction plots are shown in Figure 52. For OVA-specific $CD4^+CD25^+$ cells, there was a statistically significant difference between the treatment groups ($p = 0.04911$) with OVA specific $CD4^+$ $CD25^+$ cells being marginally higher in the C-I group compared to the I-only group.

For OVA-specific $CD8^+CD25^+$ cells, a significant effect of treatment ($p = 0.0001$) and trial ($p < 0.0001$) was identified but no treatment × trial interaction. This reflected an increase in OVA-specific $CD8^+$ $CD25^+$ cells in C-I versus I only groups in both Trial 1 and Trial 2.

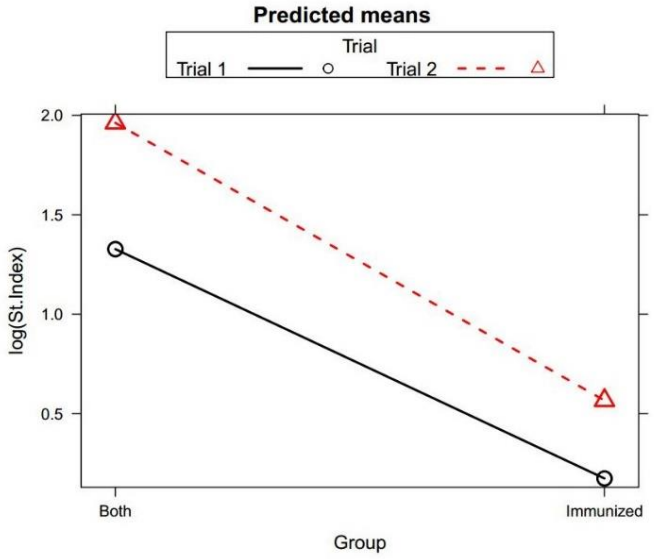


Figure 50: Interaction plot of OVA-specific PsLN lymphocyte proliferation *ex vivo* of calves from both PT21/28 Stx2c+ challenge trials. Lymph node cells were isolated post-mortem from the left hand side PsLN from calves and re-stimulated *ex vivo* for 5 days with OVA. OVA-specific lymphocyte proliferation was determined by a lymphocyte stimulation assay and is expressed as predicted mean log stimulation indices for each group. Both = challenged and OVA immunised; Immunised = OVA immunised alone.

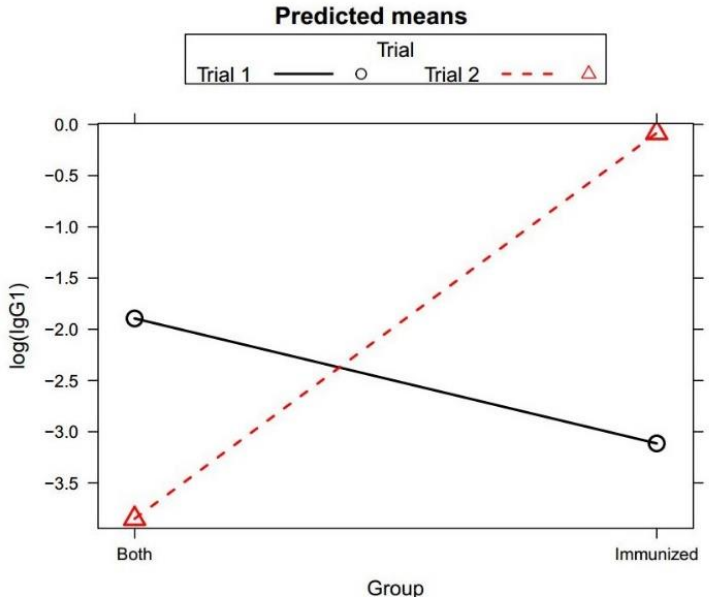
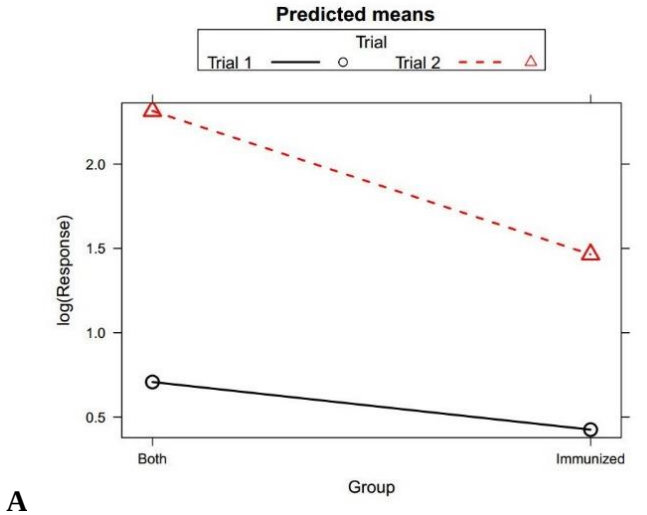
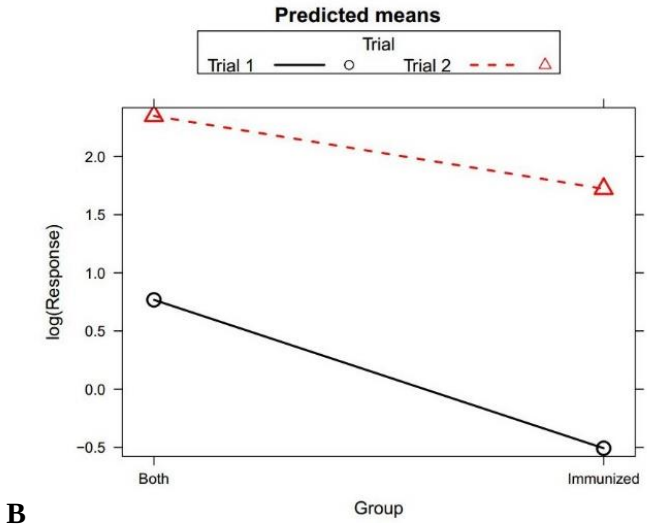


Figure 51: Interaction plot of OVA-specific IgG_1 antibody responses of PsLN lymphocytes *ex vivo* of calves from both PT21/28 Stx2c+ challenge trials. IgG_1 antibody levels were determined by ELISA and are expressed as the log of the normalised mean (of technical replicates) OD at 492 nm. The symbols represent the predicated mean of each group. Both = challenged and OVA immunised; Immunised = OVA immunised alone.



A



B

Figure 52: Interaction plots of OVA-specific $CD4^+CD25^+$ (A) and $CD8^+$ $CD25^+$ (B) cells from the PsLN of calves from both PT21/28 Stx2c+ challenge trials. OVA-specific CD4+ CD25+ cells (A) and OVA-specific $CD8^+CD25^+$ cells (B) determined by flow cytometry are expressed as log fold changes in OVA-stimulated cells compared to unstimulated controls. The symbols represent the predicted means of each group. Both = challenged and OVA immunised; Immunised = OVA immunised alone.

4.4 Discussion

The objective of this study was to characterise the effect of STEC O157 colonisation on the ability of calves to mount an immune response to a concurrently administered T-cell dependent antigen, OVA. Both systemic and local OVA-specific cellular and humoral immune responses were determined in groups of calves orally challenged with STEC O157 and subsequently immunised with OVA and unchallenged calves immunised with OVA alone. Two different wild type strains (PT21/28 Stx2c+ and PT32 Stx2c+) were used for the STEC O157 challenges and also a genetically modified isogenic version of the PT21/28 strain (Stx2a+Stx2c+) in which an insertion sequence (Isec8) was removed from the *stx2a* gene which allowed it to produce functional Stx2a. This enabled us to compare the effect on immune responses of two strains which differed only in their ability to produce Stx2a. The three challenge strains used in this study successfully colonised all of the orally challenged calves.

OVA was used for the immunisations as it is a protein known to lead to significant specific cellular and humoral immune responses in calves[208,209]. This allowed us to monitor both local and systemic immune responses and to compare them between groups of STEC O157 challenged and unchallenged control calves. In the first trial two calves in each STEC O157 challenge group received no OVA, which allowed us to determine that the OVA specific responses detected in immunised calves were OVA-specific. Furthermore, as OVA shares no homology with known O157 antigens, any OVA-specific immune response detected could be attributed to the immunisations. Also, as natural exposure of cattle to OVA is unlikely, no maternal transfer of OVA-specific antibodies was likely to occur, which could have complicated the results[217].

It has been hypothesised that it is T-cell priming that is affected by Stx and not suppression of an already established T-cell response. A study demonstrated that PBMCs from *stx*-negative *E. coli* O157 challenged calves developed proliferative responses to heat killed STEC O157 which also increased after re-challenge with a *stx2*-positive strain[126]. The same study also showed that PBMC from *stx*-positive *E. coli* O157 challenged calves did not develop consistent proliferative responses to heat killed STEC O157[126], suggesting that Stx can block induction of cellular immune responses. Since calves initially challenged with a *stx*-negative strain generated proliferative responses to heat killed STEC O157 following *stx*-positive re-challenge it was also hypothesised that the effects of Stx are limited to the

priming of cellular immune responses and do not block the recall of existing antigen specific cellular responses[126]. Thus calves in this study had their primary immunisation with OVA five days after been orally challenged with STEC O157, so we would be able to demonstrate if there was an effect on priming of the OVA immune response.

Systemic immune responses to OVA were determined by performing lymphocyte stimulation assays and bovine IFN-γ ELISpots on PBMC from the calves. IFN-γ producing cells were determined because in a previous study[97] it was demonstrated that IFN-γ release in response to Concanavalin A (a non-specific T cell mitogen) and LPS were reduced in the PsLN of calves which were orally challenged with a PT32 Stx2c+ strain of STEC O157 compared to PT21/28 Stx2c+ challenged calves and controls (unchallenged calves). Also the adjuvant Quil A used in this study has been previously shown to lead to an IFN-γ response[218,219]. There was a general trend in this study that the C-I calves, compared to the I-only calves, had lower OVA-specific systemic cellular immune responses particularly with the PT21/28 Stx2c+ challenged calves which was different to the previous study by Corbishley *et al.* (2014) when the PT32 Stx2c+ strain challenge lead to lower IFN-γ release in response to ConA compared to PT21/28 Stx2c+ challenged calves. In this study we determined OVA-specific responses compared to non-specific T-cell mitogen stimulation in the previous study, which may account for the different results. Also there were no statistically significant differences between the C-I and I- only calves in any of the systemic OVA-specific cellular responses determined in this study. Due to logistical constraints, the timing of the OVA immunisation was short, with only two immunisations given two weeks apart, and the responses were only followed for one week post the second OVA immunisation. However other studies have shown that peak circulating T and B-cell responses occur around 1 week post-immunisation in cattle, and therefore one would have expected to see differences in the systemic cellular responses during this time-frame[220].

IL-10 is an immunoregulatory cytokine promoting anti-inflammatory responses by inhibition of certain cellular responses. In infections, IL-10 suppresses macrophages and dendritic cell function, and thus limits T_H1 and T_H2 effector cell responses[221]. It has been demonstrated in bovines that IL-10 signaling leads to lack of T-cell responsiveness in the acute phase of foot and mouth disease virus infections[222]. Thus we wanted to determine if systemic OVA-specific IL-10 responses could be affected by concurrent STEC O157 colonisation in cattle. In Trial 1 there was significantly lower OVA-specific IL-10 release from *ex vivo* stimulated PBMC from PT21/28 Stx2a+Stx2c+ C-I calves compared to I-only calves. There was no significant difference with the other two strains in Trial 1, but the PT21/28 Stx2c+ C-I calves

followed the same trend. Similarly, there was no statistically significant difference in OVA-specific IL-10 between C-I calves and I-only calves detectable in Trial 2. However in Trial 2 the IL-10 values were higher in the I-only calves compare to the C-I calves, which does suggest that challenge with PT21/28 Stx2c+ reduces the IL-10 response to OVA which is potentially indicating an enhancement of the immune response.

Compared to I-only controls, OVA-specific IgG_1 serum antibody levels were significantly increased in the PT21/28 Stx2c+ C-I calves in the first trial but lower in the second PT21/28 Stx2c+ trial, indicating significant variation in the humoral immune responses. The experimental protocols, bacterial strain and antigen were identical between the two trials but calves were sourced from different farms and therefore genetic and/or other environmental effects may have played a role.

Systemic OVA-specific cellular immune responses showed some general trends implying that STEC O157 colonisation impairs such responses, although differences were not statistically significant. OVA-specific cellular responses may have been diluted by potentially low frequencies of circulating antigen-specific T-cells. Following sub-cutaneous immunisation antigen is presented in the draining lymph node and other secondary lymphoid tissue leading to an expansion of antigen-specific T-cells and re-circulation through the blood to effector sites[178,223]. Within PBMC these antigen-specific re-circulating lymphocytes can exist at very low frequencies[224], which is likely to contribute to the high levels of variation in measurements of the OVA-specific T-cell responses within the PBMC in this study. This in turn may have accounted for the lack of observable differences in cellular responses between challenged and unchallenged calves.

We hypothesised that it may be possible to determine differences between the treatment groups at the local lymph node due to increase in OVA-specific T-cells within the lymph node draining the site of immunisation. Analysis of immune responses in the left pre-scapular lymph node (PsLN) revealed a significantly enhanced OVA-specific cellular immune response in the PsLN in calves challenged with the PT21/28 Stx2c+ strain and subsequently immunised with OVA compared to I-only controls. Using CD25 as a marker of T-cell activation[225-228], it was shown that the enhanced cellular response was more pronounced for $CD8^+$ T-cells compared to $CD4^+$ T-cells. No similar enhancement in cellular immune responses were seen when calves were challenged with the other two STEC O157 strains.

In contrast to the enhancement of local cellular immune responses, local antibody responses were more variable: with the PT21/28 Stx2c+ strain, production of OVA-specific IgG_1 was

enhanced in C-I calves compared to I-only controls in Trial 1 but the opposite was observed in Trial 2. The effects on B-cell responses in this study seem to be much less clear than any effect on T-cell responses, an observation which is consistent with another *in vivo* cattle study which indicates that T-cells are more affected by Stx than B-cells[126]. As OVA is a T-cell dependent antigen and IgG_1 is a class switched antibody[169], OVA-specific IgG_1 responses are dependent on $CD4^+$ T-cell help. Therefore differences in the effects of challenge on the local OVA-specific IgG_1 response between the two trials may relate to differences in the response of B-cells and/or $CD4^+$ cells. CD4 responses appeared to be enhanced in PT21/28 Stx2c+ challenged calves in both Trial 1 and Trial 2, suggesting that the differences in IgG_1 responses were not due to differences in T-cell help. However, the OVA-specific cytokine release by local immune cells was more variable. There was a statistically significant difference in OVA-specific IFN-γ release in Trial 1 PT21/28 Stx2c+ challenge, but this was not repeated in Trial 2. There was no statistically significant difference in OVA-specific IL-10 release in Trial 1 with the PT21/28 Stx2c+ challenge but we did determine a statistically significant difference between the C-I and I-only calves in Trial 2 with the PT21/28 Stx2c+ challenge. The I-only calves had higher OVA specific IL-10 levels than the C-I group. This agreed with the systemic OVA-specific IL-10 responses, both suggesting that STEC O157 challenge downregulates IL-10 responses, suggesting an enhancement effect on immunity.

The differences between trial 1 and trial 2 with regards to OVA-specific IgG_1 responses both systemically and locally were not seen with the EHEC specific antibody responses. The H7, Tir, Intimin and EspA specific antibody responses both systemically and locally were determined for the calves in both trial 1 (Chapter 3, Figures 27-31) and trial 2 (data not shown), the results were very similar for both trials with no contrasting results between the two trials as seen with the OVA specific IgG_1 responses. This indicates that these opposing results are not generalised changes in antibody responses between calves in the two trials.In this study the PT21/28 Stx2c+ challenged animals showed a significant increase in OVA-specific cellular responses in the local PsLN which was not demonstrated with the isogenic PT21/28 Stx2a+Stx2c+ strain. The wild-type PT21/28 Stx2c+ strain has an insertion sequence Isec8 within the *Stx2a* A subunit (which has been genetically removed from the PT21/28 Stx2a+Stx2c+ strain), which prevents the generation of functional Stx2a, as determined by a lack of *in vitro* verocytotoxicity (Dr Stephen Fitzgerald, personal communication). PCR analysis using primers specific to the B-subunit of *stx2a* and ELISA specific for the Stx2 B-subunit demonstrate, at least *in vitro*, that the B-subunit of *Stx2a* gene is expressed at both the gene and protein level (Dr Stephen Fitzgerald, personal

communication). The B-subunit of Stx has been shown to act as an adjuvant via direct interaction with antigen-presenting cells[229,230].

The genes for the A and B subunit of Stx are carried in the late region of lysogenic bacteriophages[49]. Once the bacterial SOS stress response triggers viral lytic replication of the bacteriophage, Stx transcription starts. A recent paper has demonstrated that the A and B subunit are not released as a holotoxin and that pre-assembly is not required for toxicity[231]. It is also believed that Stx, especially Stx2a, unlike other AB5 toxins, including cholera, have a decreased tendency to maintain an intact AB5 conformation. This seems to be due to a small hydrophobic patch in the central pore of the B pentamer which in other AB5 toxins is larger and plays a critical role in the assembly of the A subunit with the B pentamer[232]. In the absence of Stx A subunit the Stx B subunit can still adopt a pentameric structure that is functionally equivalent to the holotoxin in receptor binding[233]. The B subunit binds to the CD77 receptor initiating endocytosis and if the A subunit is present this leads to inactivation of protein synthesis and thus cell death. A study in mice has shown that the B-subunit of Stx1 can act as an adjuvant to subcutaneous OVA immunisations by eliciting antigen specific CD4 T_H1 and T_H2 type responses and the subsequent induction of antigen-specific IgG_1 and IgG_{2a} antibody responses[229]. Another study in mice using a StxB-OVA recombinant protein to stimulate splenocytes and then co-incubate with OVA-specific $CD4^+$ T cells demonstrated both IL-2 and IFN-γ secretions were enhanced, indicating that a T_H1 type response was induced by the B subunit[230]. It is possible that the enhanced OVA-specific cellular immune response seen with the wild type PT21/28 Stx2c+ strain in this current study is due to production and dissemination of the Stx2a B-subunit without an active (and toxic) A-subunit. This would explain the lack of any adjuvant effect with the repaired PT21/28 strain as any Stx2a B-subunits would have an active A subunit, which would mitigate any immunostimulatory effects of the B-subunit via direct cytotoxic effects.

This study indicates that colonisation with STEC O157 can alter local adaptive immune responses to a non-bacterial antigen, but this immunomodulation is strain dependent. Corbishley *et al.* (2015) previously demonstrated temporal differences in both STEC antigens (T3SPs) and non-antigen specific cellular responses (ConA) between the two STEC O157 wild type strains (PT21/28 Stx2c+ and PT32 Stx2c+) used in this study, again demonstrating the variable effect of immune regulation with different *stx* positive STEC O157 strains. This study demonstrates the variation in cellular and humoral immune responses to immunisations in individual cattle. Immunisations against a variety of bacteria and virus in the field demonstrate variable immune responses in individual cattle and it is

frequently the herd response that enables the vaccine to work efficiently[188,234]. In this study a large bolus doses of STEC O157 were used to challenge the calves while natural infections in the field will involve intake of low doses over a longer period of time. Even with this large dose any differences seen in the immune responses to OVA in cattle challenged with STEC O157 were marginal and only demonstrated consistently at the local site of immunisation and not systemically. We hypothesised that STEC O157 would lead to suppression of OVA immune responses and in fact the only consistently significant results were an enhancement in the immune response. Others have previously hypothesised that any suppression of immune response by Stx is not generalised[126], however contrasting results have previously indicated that the suppression may be more generalised[97]. Colonisation of cattle by different strains of STEC O157 may be contributing to some of the natural variation in individual animals we appreciate with immune responses to routine vaccinations and infections, but from this study it seems unlikely that it is having a significant effect in the cattle population.

Chapter 5

Transcriptomic analysis of immune genes at the terminal rectum following experimental STEC O157 challenge

5.1 Introduction

Cattle are the main reservoir of STEC O157:H7 and it is known that the recto-anal junction (RAJ) is the primary site of colonisation within the bovine gastro-intestinal tract[27]. Colonisation of the rectal epithelium is thought to be initiated by the bacterial flagella (H7 flagella) which form physical contact points with the rectal epithelial cells[235]. Once anchoring of the bacteria to the epithelium has occurred, flagellar expression is down-regulated and other bacterial adhesion mechanisms such as the T3SS takes over. The T3SS is essential for colonisation of cattle as deletion of the *LEE4* operon, which encodes for several T3SS proteins including the Esps (EPEC secreted proteins) A, B, D and F, SepL and EscF, completely abrogates colonisation[32]. Cattle which are either experimentally or naturally colonised with STEC O157 are able to control the infection over a number of days or weeks down to shedding levels undetectable by PCR and culture at several sampling points; however, the exact mechanism by which bacteria are cleared from the epithelium is unclear.

As discussed in Chapter 2, antibodies against a number of STEC antigens are induced following exposure[116,118,175]; however, the presence of these antibodies is poorly correlated with protection against repeated STEC O157 exposure[236,237]. A recent study has shown that oral STEC O157 challenge results in an up-regulation of transcription of T_H1 associated cytokines within the rectal mucosa and an increase in the proportion of $CD4^+$ T-cells within the rectal lymph nodes, indicating that cattle mount a local cellular immune response during colonisation with STEC O157[97]. $CD4^+$ T-cells from this same study have been shown to recognise specific STEC O157 antigens[179]. This study also demonstrated that the temporal nature of the immune response differed depending on the challenge strain[97]. Therefore, it is

clear that STEC O157 induces local adaptive immune responses at the primary site of infection although it is unclear how much it contributes to bacterial clearance or protection from re-infection.

Cattle shedding more than 10^4 CFU STEC O157 per gram of faeces are defined as super shedders[5] and it is believed that these animals are the source of most of the bacteria that contaminate the environment and enter the food chain[15]. It is largely unknown why some animals become super shedders and some only shed at low levels and apparently are able to quickly clear the bacteria; factors involved are hypothesised to be bacterial strain, host genotype, host animals management/ environmental factors and gastrointestinal microbiome of the host animal[238]. Some strains of STEC O157 (for example containing specific phage-types) are more likely to be associated with super shedding than others[5,28]. Host animal's diet has been linked with super shedding; however, changes in diet do not consistently correlate with faecal shedding in cattle[89,90,92]. Other environmental factors such as rainfall, relative humidity and pasture growth have been shown in one longitudinal study to be positively associated with increased shedding in heifers at pasture[239]. Farms with cows, young animals, and concentrated calving periods all appear to be at more risk of observing a high-level shedder[5]. The host genotype is also likely to be a contributing factor to shedding levels and the Scottish IPRAVE study determined that female breeding cattle were more likely to be associated with high levels of shedding[5], although it is difficult to determine if this is just a gender effect as these animals will not only be different genders but also kept in different conditions to male cattle kept for meat production. There have also been links between STEC O157 colonisation in cattle and the nature of the microbial community that inhabits the digestive tract of super shedding animals[240]. However a recent study has demonstrated that the overall bacterial community structure was not associated with STEC O157 shedding status, although the authors did demonstrate that several of the major bacterial genera were differentially abundant in super shedding verses non-shedding cattle, although interestingly not at the terminal rectum[241].

A previous study by Wang *et al.* (2016) has identified 47 genes with lower levels in the terminal rectum of naturally infected super shedding cattle by RNA sequencing of rectal mucosal biopsies taken at post mortem[129]. Of these, 31 genes were associated with innate and adaptive immune functions, 10 of which were selected for qPCR validation. Expression of seven chosen genes (CD22, IL2RA, LTB, CCR7, CD19, POU2AF1, CXCL13) were significantly lower in super-shedding compared to non-shedding cattle, the transcription of the other three genes (MS4A1, CCL21, SH2D1A) followed the same trend, being lower in

the super-shedding cattle[129]. The major limitation of the study was that only one sampling time point was analysed, and therefore it is unclear whether the lower level of immune genes was due to immunosuppressive effects of the bacteria during colonisation, or represented an inherent deficiency in rectal immune function in the super-shedding individuals which in turn increased their susceptibility to colonisation and super shedding.

The 10 immune gene transcripts with lower levels in super shedding cattle that Wang *et al*. (2016) validated by qPCR are listed in Table 12 and the following briefly describes their function relating to the immune system.

Membrane spanning 4-domains, subfamily A, member 1 (MS4A1) encodes for CD20, which is expressed on the surface of B-cells and plays a role in the development and differentiation of B-cells into plasma cells (it is not expressed on plasma cells)[242]. MS4A1 enables optimal B-cell immune response, specifically against T-independent antigens[243].

CCL21 a chemokine, is expressed in various lymphoid tissues and activates B and T lymphocytes. CCL21 is a chemoattractant for B-cells and regulates B-cell movement in secondary lymphoid tissues[244], and also leads to migration of dendritic cells[245] and T-cells[244].

SH2D1A (SH2 domain protein 1A) in humans is expressed on activated T-cells and NK cells but not on macrophages or activated B-cells[246].

CD22 acts as an accessory component of the B-cell receptor (BCR), is expressed on the surface of mature B-cells and acts to help to control over-activation of the immune system[247 248].

Interleukin 2 receptor alpha (IL2RA), also known as CD25, is up-regulated on activated T-cells[249]. Interleukin 2 (IL-2) is produced by activated T_H1 cells; it stimulates cell proliferation, antibody and IFN-γ production and enhances cytotoxicity[250,251]. IL-2 also supports the survival of regulatory T-cells and is thus important in the regulation of immune responses[252].

Lymphotoxin beta (LTB) is also known as Tumour necrosis factor C and is expressed by mature B, T and NK cells[253,254]. LTB is an inducer of the inflammatory response system and involved in the normal development of lymphoid tissue. Decreases in LTB leads to a reduction in the number of lymphoid follicles in the gastrointestinal tract in mice[255].
Chemokine receptor 7 (CCR7) is involved in homing of T-cells to lymph nodes and other secondary lymphoid organs[256]. When dendritic cells are activated in peripheral tissue they

express CCR7 on their surface[257]. CCR7 recognises CCL19 and CCL21 which are produced in lymph nodes, guide the migration of the activated dendritic cells into the lymph nodes and stimulate dendritic cell expression of MHC class I or Class II[245].

CD19 is a B-cell specific molecule and, like CD22, positively regulates B-cells activity; through complexing with the BCR[258 259], CD19 also regulates TLR B-cell activation[259].

POU class 2 associating factor 1 (POU2AF1), also known as OCT-1, is involved in B-cell proliferation and germinal centre formation[260].

Chemokine ligand 13 (CXCL13) is a small cytokine and a chemoattractant of B-cells. Expression of this gene is required for migration of B-cells to gut associated tissues and formation of lymphoid follicles[261,262]. CXCL13 elicits its effects by interacting with the chemokine receptor CXCR5[263]. A study by St John *et al.* (2009) in mice demonstrated that following experimental infection, via injections of *Salmonella typhimurium* into the footpads and consequently trafficking of *S.typhimurium* through the draining lymph node (popliteal lymph node), there was down-regulation of CXCL13 and CCL21 in the popliteal lymph node[264]. The *S. typhimurium* disrupts the lymph node architecture and cellular trafficking, which enhances its virulence and this could serve as a mechanism of immune suppression used by other pathogens that primarily target lymphoid tissue[264].

All these genes of interest are directly related to immune function and down-regulation could allow bacteria to survive and colonise more successfully at the terminal rectum.

Another recent study using an experimental STEC O157 challenge concluded that genes associated with immune responses in the ileal peyer's patches were more influenced by STEC O157 infection in comparison to the recto-anal junction[130]. In this study calves were challenged once or twice with a *stx*-negative strain of *E. coli* O157 and samples of ileal and rectal mucosa collected for RNA sequencing, with results compared to non-challenged controls. This study identified significantly more differentially expressed genes in the ileum compared to the recto-anal junction following primary infection (1159 and 15 differentially expressed genes for ileum and recto-anal junction, respectively) but a similar number of differentially expressed genes at both sites following secondary infection (17 and 10 genes for ileum and rectum, respectively). Of the differentially expressed genes, 69 immunostimulatory genes were down-regulated and 7 immunosuppressive genes were up-regulated in the STEC O157 colonised calves. One major limitation of this study was that the primary-infected calves were not-weaned, whereas the secondary challenged calves and

unchallenged controls were both older and weaned. Therefore the differences between primary challenged and control calves may largely be due to age and/or diet effects[265-267]. Nevertheless, consistent with the Wang study, these data suggest that STEC O157 colonisation is associated with down-regulation of mucosal immune responses.

Table 12: Immune genes with lower levels in super shedding cattle taken from Wang *et al.* (2016) and modified.

Gene	General Function	Fold change (log 2)*	Validated by qPCR
MS4A1 (membrane spanning 4-domains, subfamily A, member 1)	Important for B cell proliferation[268]	-2.9	Not Significant
CCL21 (C-C motif ligand 21)	Chemoattractant for B cells[244]	-3.4	Not Significant
SH2D1A (SH2 domain protein 1A)	Decrease activation of NK cells[269]	-2.2	Not Significant
CD22 (CD22 molecule)	Decrease in T cell differentiation, B-cell membrane protein[247]	-2.8	Significant
IL2RA (Interleukin 2 receptor alpha)	IL2 receptor	-2.7	Significant
LTB (lymphotoxin beta)	Decrease in lymphotoxin-decrease in lymphoid follicles in the GIT[255]	-2.9	Significant
CCR7 (Chemokine receptor 7)	Cytokine-cytokine interaction and chemokines[270]	-3.6	Significant
CD19 (CD19 molecule)	B-cell membrane protein, promotes B-cell activation[259]	-2.6	Significant
POU2AF1 (POU class 2 associating factor 1)	B cell proliferation and germinal centre formation[260]	-2.0	Significant
CXCL13 (Chemokine ligand 13)	Chemoattractant for B cells, essential for migration in GIT[261]	-4.3	Significant

*log 2 (fold change) is log ratio of gene expression level in super-shedders to non-shedders.

A study using a bovine-specific cDNA microarray to analyse rectal biopsy specimens taken 7 days post rectal challenge with STEC O157 demonstrated down-regulation of 49 genes in

response to colonisation with STEC O157 across a range of different pathways[271] and also up-regulation of 22 genes. None of the gene transcripts which were determined to be up or down-regulated 1 or 7 days after rectal challenge with STEC O157 were the same genes as the gene transcripts found to be differentially expressed in the Wang *et al.* (2016) study. Four of the genes down-regulated at day 7 were believed to be directly associated with immune function. Unfortunately because a subset of 1,676 genes were present on the microarray and the list of genes is no longer available we cannot determine if the genes in the Wang *et al.* (2016) study were analysed or not.

This current study aims to aid in the further investigation of suppression of gene transcripts related to immune function at the terminal rectum in *stx* positive STEC O157 colonised calves. Importantly repeated rectal biopsies (pre, peak and post challenge) were taken from calves who were experimentally orally challenged with STEC O157, allowing us to determine if there is a temporal down-regulation of the immune genes identified by Wang *et al.* (2016) during STEC O157 colonisation which would be suggestive of active immune suppression by the bacteria. Two other genes, CD80 and CD86, which are key genes for adaptive immune induction were also analysed. CD80 and CD86 work in tandem to prime T-cells[272]. CD80 is present on the surface of dendritic, activated B-cells and monocytes[273] and provides a co-stimulatory signal necessary for T-cell activation and survival[274]. CD86 is expressed on antigen presenting cells and provides the co-stimulatory signals with CD80 necessary for T-cell activation and survival[274,275]. As many of the genes determined to be downregulated by Wang *et al.* (2016) are directly involved in T-cell activation, we anticipated that these two genes involved in the induction of the adaptive immune response may also be affected by STEC O157 colonisation.

Main Aim

To quantify transcripts for specific genes involved in immunity at the terminal rectum of cattle during colonisation of cattle with three different strains of STEC O157.

5.2 Materials and Methods

5.2.1 Experimental animal challenge trials

See chapter 2 material and methods for the challenge animal trials involving calves orally challenged with three different STEC O157 strains (PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+).

5.2.2 Rectal Biopsies

Rectal biopsies were taken from challenged and control calves pre-challenge (day -3), peak (day 7) and post challenge (day 26). A local anaesthetic was applied to the anal sphincter (5 % EMLA, Astra Zeneca, UK), and a rectal speculum was applied to allow visualisation and access to the rectal mucosa. Pinch biopsies were taken approximately 5 cm proximal to the recto-anal junction; biopsies were taken from two opposing sites at each time point. Different sites of rectal mucosa were biopsied at each time point. Biopsies were put into RNA later (Ambion, Paisley, UK) at 4 ^{o}C overnight and then stored at -80 ^{o}C. For long term storage the biopsies were removed from the RNA later and stored at -80 ^{o}C.

5.2.3 cDNA preparation

RNA was extracted from the rectal biopsies using QIAshredder kit (Qiagen, Germany) and then the RNeasy Plus mini kit (Qiagen, Germany). Biopsies (weighing 20-30 mg) were initially disrupted in Precellys CK28 tubes (Stretton Scientific, UK) using 3 × 23 second cycles at 5,800 revolutions per minute (rpm) in a tissue homogeniser (Percellys24, Bertin Instruments, France). Tubes were placed on ice for 2 minutes between cycles. Residual DNA was digested on the RNeasy Plus mini kit column with DNase I (Qiagen, Germany).

RNA yield was estimated using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Waltham, USA). The ratio of absorbance at 260 nm and 280 nm was used to help assess the purity of RNA and also the 260 nm and 230 nm absorbance; RNA was only used if both ratios were > 1.8. RNA quality was further assessed using a RNA 6000 Nano total RNA kit

and 2100 Bioanalyzer (Agilent Technologies, Wokingham, UK). All RNA samples used to make cDNA had a RNA integrity number (RIN) above 7.0. A number of samples with low absorbance ratios as per the nanodrop measurement were run through a second RNeasy Plus mini column (Qiagen) and cleaned up as per the manufacturer's instructions.

cDNA was synthesised as per manufacturer's instructions using Superscript II (Invitrogen, UK) reverse transcriptase, RNAseOUT (Invitrogen), dNTP mix (Invitrogen) and Oligo (dT)-23 primers (Sigma-Aldrich, UK) from 2 µg RNA, where 1 µg was pooled from each of the two biopsies taken at each time point. Reaction volume was 40 µl. Cycling conditions were 42 ºC for 2 minutes followed by addition of reverse transcriptase, 42 ºC for 50 minutes and 70 ˚C for 15 minutes. cDNA was diluted 1 in 10 using nuclease-free water and stored at -20 ºC prior to use.

Table 13: Primers used for reverse transcriptase quantitative PCR analysis of gene expression and sequencing of plasmid inserts.

Gene	Direction	Sequence	Reference
CCL21	F	GCTATCCTGTTCTCGCCTCG	Wang *et al*. (2016)
CCL21	R	ACTGGGCTATGGCCCTTTTG	Wang *et al*. (2016)
CCR7	F	ACCCTCGCTAGCTACCTCAA	Wang *et al*. (2016)
CCR7	R	CGGTCTCTTGTCTTGGGGAC	Wang *et al*. (2016)
CD22	F	ACCTCAGTTTCCAGCCCAAG	Wang *et al*. (2016)
CD22	R	CCTCATGGTCACAGACTCGC	Wang *et al*. (2016)
CXCL13	F	AACCCTCAAGCCAAATGGACA	Wang *et al*. (2016)
CXCL13	R	AACCCGGAGCAGGAATGTTG	Wang *et al*. (2016)
LTB	F	TGGGAAGAGGAGGTCAGTCC	Wang *et al*. (2016)
LTB	R	TAGCTTGCCATAAGTCGGGC	Wang *et al*. (2016)
MS4A1	F	GCGGAGAAGAACTCCACACA	Wang *et al*. (2016)
MS4A1	R	GGGTTAGCTCGCTCACAGTT	Wang *et al*. (2016)
IL2RA	F	GCACGGTCAGGCTTCAGAT	Wang *et al*. (2016)
IL2RA	R	TTCTTGACTTCTTCTGGCCTTG	Wang *et al*. (2016)
CD19	F	CTCCCATACCTCCCTGGTCA	Wang *et al*. (2016)
CD19	R	GCCCATGACCCACATCTCTC	Wang *et al*. (2016)
POU2AF1	F	GAGACCATGGTGACTGGTGG	Wang *et al*. (2016)
POU2AF1	R	AATACGGCCATTGTGGGGAG	Wang *et al*. (2016)
SH2D1A	F	CAGCACCGGGGGTACATAAA	Wang *et al*. (2016)
SH2D1A	R	TCCTGTAGCACCTTGTGTACTT	Wang *et al*. (2016)

Table 13 continued: Primers used for reverse transcriptase quantitative PCR analysis of gene expression and sequencing of plasmid inserts

Gene	Direction	Sequence	Reference
CD80	F	CTGTGATTACAACACGACCACTGA	Peimin Lio Personal communication (Edinburgh University, UK)
CD80	R	ATGGTGCGGTTCTCGTATTCA	Peimin Lio Personal communication
CD86	F	GCCAAGAGAAGCCCAATAACG	Peimin Lio Personal communication
CD86	R	CAGTCCTTGGGACCTTCTATCATG	Peimin Lio Personal communication
T7	F	TAATACGACTCACTATAGG	Eurofins (Paris, France)
Sp6	R	ATTTAGGTGACACTATAG	Eurofins (Paris, France)

5.2.4 Quantitative PCR

Unless stated otherwise quantitative PCR reactions were conducted in duplicate in 96 well qPCR plates using SYBRgreen ER qPCR super mix (Invitrogen) with ROX and an ABI prism 7500 qPCR instrument (Applied Biosystems, Paisley, UK) as per manufacturer's instructions. The reaction volume was 20 µl. Cycling conditions were 50 ^{o}C for 2 minutes, 95 ^{o}C for 10 minutes and then 40 cycles of 95 ^{o}C for 15 seconds, 60 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds, with the exception of the IL2RA PCR in which cycling conditions used were 50 ^{o}C for 2 minutes, 95 ^{o}C for 10 minutes and then 40 cycles of 95 ^{o}C for 30 seconds, 60 ^{o}C for 60 seconds. Melting curve analysis was performed at the end of each PCR run to confirm the specificity of the reactions. Primers and standard curve plasmids for GAPDH and ATP5B were supplied by Primer Design and used as per manufacturer's instructions. All other primers were supplied by Eurofins genomics (Acton, UK; see Table 13 for primer sequences).

5.2.5 Reference gene selection

Reference genes were selected using the bovine GeNorm kit 9 primer design containing primers ATP5B, EIF2B2, ACTB, SDHA, RPL12 and GAPDH as per manufacturer's instructions, qPCR reactions were prepared using 5 µl cDNA from 3 time points for eight animals (4 controls and 4 challenged calves). qBasePLUSv2.4 (Biogazella, Zwijnaarde, Belgium) software was used to select GAPDH and ATP5B as the most stably expressed genes. C_T values were converted into relative quantities for analysis with GeNorm, considering the PCR efficiencies of the genes. The program selects from a panel of candidate reference genes the two most stable genes or a combination of multiple stable genes for normalization. The stability value is based on the combined estimate of intra- and intergroup expression variations of the genes studied.

5.2.6 Plasmid generation

PCR primers for the genes of interest were previously designed by Wang *et al.* (2016) or within the group (CD80 and CD86) see Table 13. Gene fragments were generated using Go Taq G2 DNA polymerase (Promega, UK), dNTPs (Invitrogen) and bovine cDNA from a rectal biopsy as per manufacturer's instructions. Cycling conditions were 95 ^{o}C for 2 minutes, 35 cycles of 95 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds, 72 ^{o}C for 1 minute and then 72 ^{o}C for 5 minutes. Samples were run on a 1 % agarose gel with gel red detection to confirm a product of the correct size. QIAquick PCR purification kit was used (Qiagen, Germany) to purify the PCR product. The amount of product was then estimated using Nanodrop ND1000 spectrophotometer (Thermo Scientific) and then ligated into pGEM-T Easy vector plasmids (Promega) as per manufacturer's instructions.

XLI blue, Ultracompetent cells (Agilent technology, UK) were transformed with the plasmids as per manufacturer's instructions. X-gal and IPTG were used to screen colonies. Two colonies were picked for each gene of interest, and PCR performed as above (using the primers for the gene of interest) to check for a correct size insert. One PCR positive colony for each gene of interest was inoculated into 10 ml LB broth and incubated at 37 ^{o}C overnight. QIAprep Spin Miniprep kit (Qiagen) was used as per manufacturer's instructions to purify the plasmids. Plasmids were then sent to Eurofins Genomics for sequencing using

the T7 and Sp6 primers (Table 13). Sequences were checked against the *Bos taurus* Refseq RNA database using BLAST (NCBI) and primers for the gene of interest using EMBL-EBI cluster analysis tool. DNA concentrations were estimated using Nanodrop ND1000 spectrophotometer and diluted using PCR water to 10^9 copies per µl and stored at -20 ^oC until required.

Plasmids were diluted across the dynamic ranges indicated in Table 14 and used to generate a standard curve for each gene on each qPCR plate run.

Table 14: qPCR standard curve dynamic range

Gene	Regression range R^2	Gene copies/well Top standard	Gene copies/well Bottom Standard
CCL21	0.993-0.998	10^8	10^3
LTB	0.99-0.999	10^8	10^3
CXCL13	0.997-0.999	10^8	10^2
CCR7	0.992-0.999	10^8	10^3
CD19	0.994-0.999	10^8	10^2
CD22	0.992-0.997	10^8	10^2
POU2AF1	0.99-0.998	10^8	10^2
MS4A1	0.996-0.999	10^8	10^2
SH2D1AF	0.99-0.996	10^8	10^2
IL2RA	0.998-1	10^8	10^3
CD80	0.991-0.998	10^8	10^3
CD86	0.988-0.993	10^8	10^2

5.2.7 Copy number calculation

Standard curves were calculated from the cycle threshold (C_T) values using Prism 7500 SD software v1.2.3 (Applied Biosystems) and the calculated gene copies/ µl cDNA. An

arithmetic mean for the technical repeats was calculated and then each gene was normalised to the geometric mean of the reference genes ATP5B and GAPDH as previously described[276]. Briefly a normalisation factor was calculated based on the expression level of the two chosen housekeeping genes. For accurate averaging of the control genes, the geometric mean was used instead of the arithmetic mean as it controls better for possible outlying values and abundance differences between the different genes[276].

5.2.8 Statistical analysis

Statistical analysis was performed using R version 10 by Javier Palarea (Biomathematics and Statistics; Scotland; BioSS). RT-qPCR data was used to compare changes in gene expression in challenged vs. control treatment groups across 12 genes. Results for each STEC O157 challenge strain group were analysed separately together with their respective unchallenged controls. Fold changes in gene copy numbers from pre-challenge levels over the two time points (7 and 26 days post challenge) were $\log_{10}$ transformed and linear mixed models (LMMs) were fitted, with $\log_{10}$ transformed pre-challenge gene copies, treatment group, days post challenge and the interaction between group and days post challenge were fixed effects and animal was fitted as a random effect. Statistical significance was concluded at a *p*-value < 0.05.

5.3 Results

5.3.1 Faecal shedding data

Figure 24 in chapter 3 shows the faecal shedding data for the orally challenged calves, all control calves were STEC O157 negative throughout the trial (data not shown).

5.3.2 Reference gene selection

The stability of six reference genes was assessed using the GeNorm algorithm[276] incorporated in the qBasePLUS software package by comparison of the C_T values for these genes across a representative sample of reverse transcription reactions. The GeNorm M value indicates the stability of expression of the genes[276]. Figure 53 indicates the GeNorm M value for the 4 most stable genes, and indicates that ATP5B and GAPDH are the most stable genes.

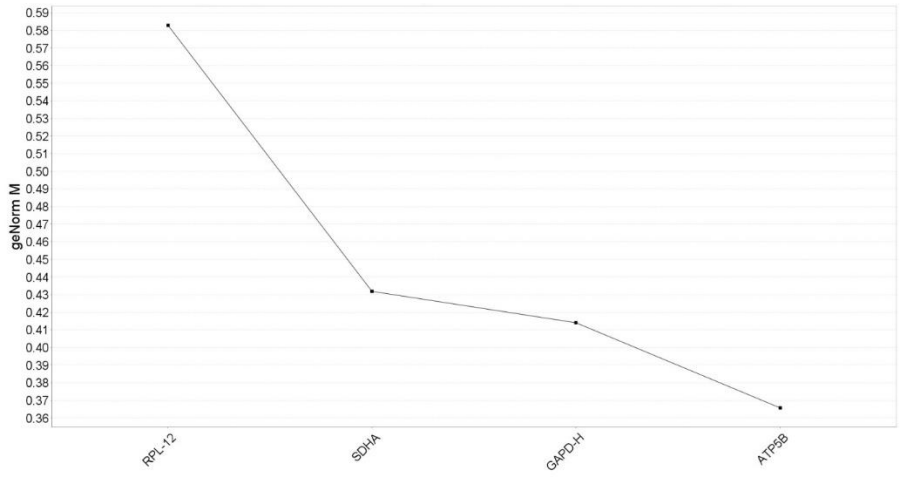


Figure 53: Indicates the average expression stability of the four most stable reference genes used to determine the suitable reference genes for the qPCR.

The optimal number of reference genes was selected using the GeNorm V values indicated in Figure 54.

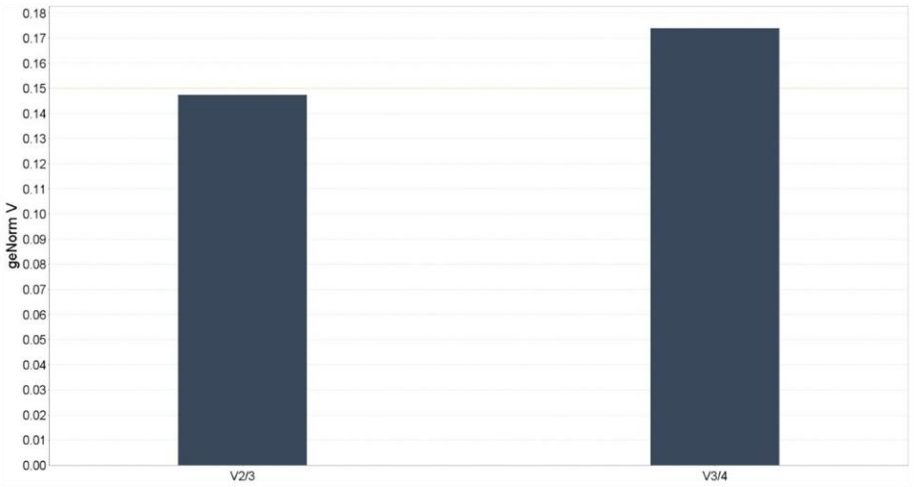


Figure 54: The GeNorm V values calculated to determine the optimal number of reference genes for the study.

The optimal number of reference targets was 2 (GeNorm V < 0.15) when comparing a normalisation factor based on the 2 or 3 most stable targets. As such, the optimal normalisation factor could be calculated as the geometric mean of reference targets GAPDH and ATP5B.

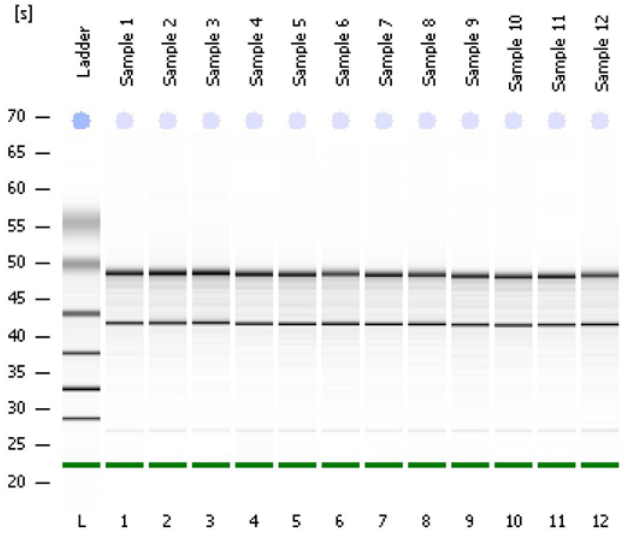


Figure 55: An example of electrophoresis of RNA samples using the BioAnalyzer. All samples run on this chip were acceptable.

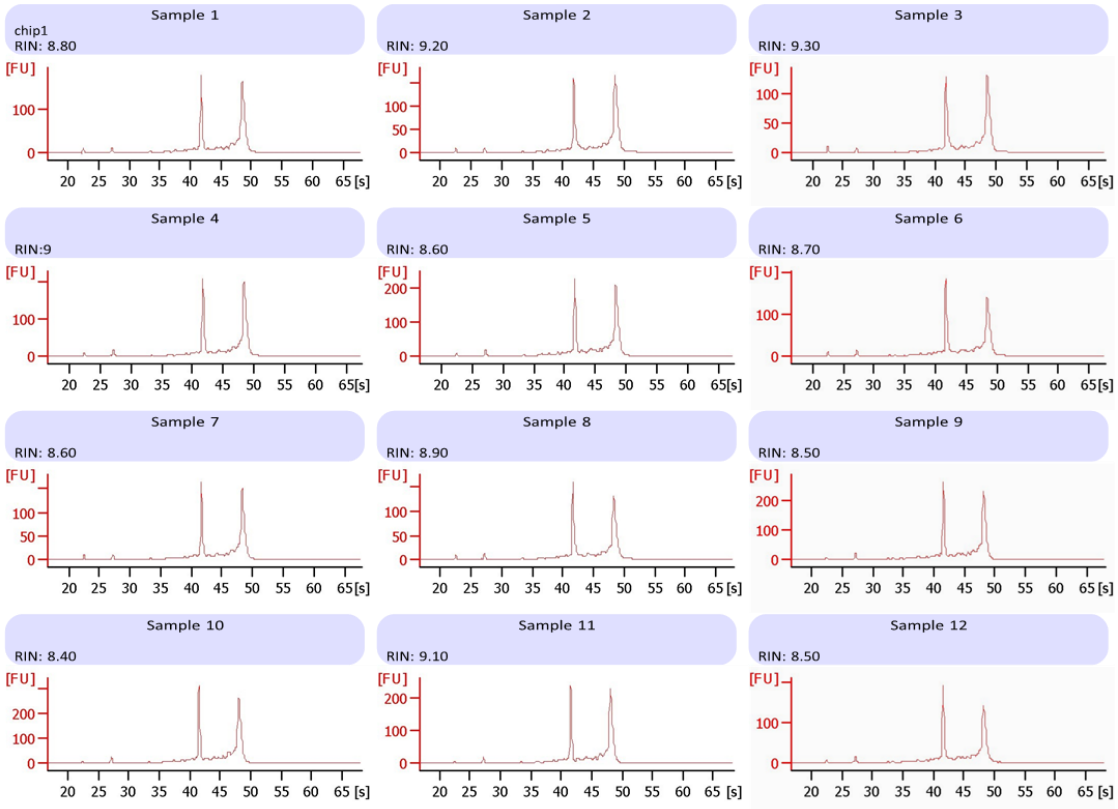


Figure 56: RNA integrity numbers and graphs of 12 RNA example samples extracted from rectal biopsy tissue. RIN calculated using the BioAnalyzer.

5.3.3 RNA extraction

Figure 55 and 56 are representative examples of bioanalyzer runs on a 12 sample chip. All RNA samples used to generate cDNA had a RIN value 7.0 or above.

5.3.4 Gene expression at the terminal rectum

The numbers of transcripts for specific immune genes were compared between STEC O157 challenged and unchallenged calves. Three different strains of STEC O157 were used for challenge (PT21/28 Stx2c+, PT32 Stx2c+ and PT21/28 Stx2a+Stx2c+), and comparisons were only made within trial (i.e. not between different STEC O157 strains). As there was significant variation in transcript levels between calves at the pre-challenge time point, changes in gene expression were determined within each individual by comparing fold-changes in transcript levels relative to pre-challenge levels.

5.3.5 Gene expression following PT21/28 Stx2c+ challenge

There was a general trend across ten of the immune genes (CCL21, CCR7, CD19, CD22, CXCL13, IL2RA, LTB, MS4A1, POU2AF1 and SH2D1A) for the unchallenged calves to have a fold increase in gene transcripts at 26 days compared to the challenged group (Figure 57). However there was often significant variation within the groups, with overlapping 95 % Confidence Intervals (CI) predicated from the linear mixed model (the predicted means and CI from the linear mixed model are represented in Figure 60 in the Appendix and Table 18 in the Appendix). There was no statistically significant difference found between the treatment groups (challenged vs. unchallenged calves) for any of the ten genes listed above (see Table 15 in Appendix for *p*-values). Although some of the graphs show diverging slopes for these genes especially from day 7 to day 26 between the challenged and control group, again no statistically significance of the treatment group nor treatment × time interaction effect was found for any of the 10 genes (see Table 15 in Appendix for *p*-values).

The fold change from pre-challenge in transcripts for CD80 and CD86 showed a different pattern. CD80 showed an increase in the challenged animals at day 7 but by day 26 both

treatment groups had returned to pre-challenge levels. There was no statistically significant difference between the treatment groups nor any treatment × time interaction; however there was a statistically significant difference in CD80 levels over time (independent of challenge; $p = 0.0003$). CD86 fold change in transcript levels were close to 1 at day 7 and day 26 for both challenged and unchallenged controls. Again there was no statistically significant difference between the treatment groups, or any treatment × time interaction.

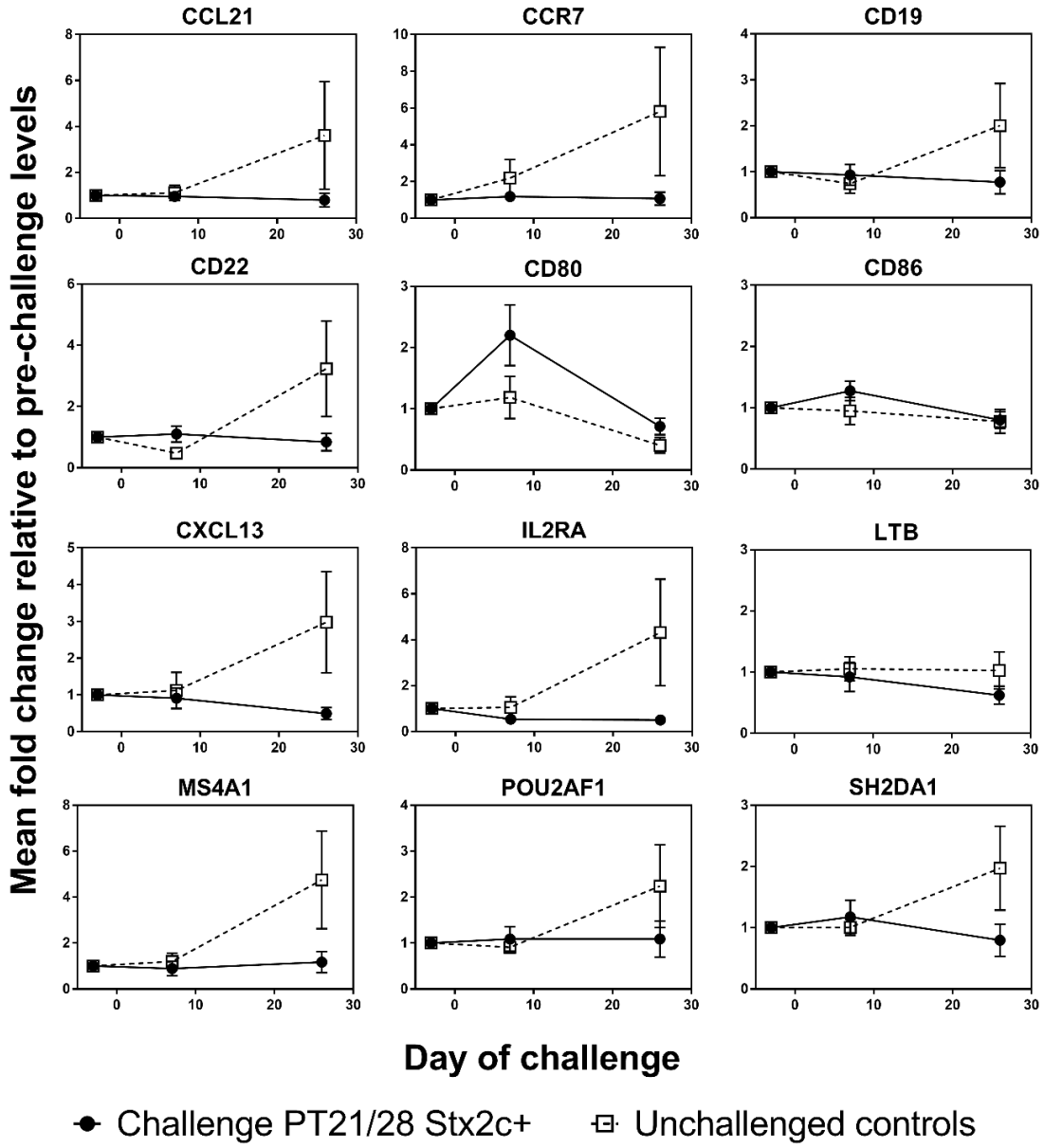


Figure 57: Fold change relative to pre-challenge, gene expression at the terminal rectum of calves orally challenged with STEC O157 PT21/28 Stx2c+. The graphs represent mean fold changes (± standard error of the mean, SEM) in gene copy numbers relative to the pre-challenge (day -3) gene copy numbers at day -3, 7 and 26 for each gene considered. The gene copy number is calculated using a plasmid standard for each gene and normalisation to two housekeeping genes. The black circles represent the mean of the challenged calf group and the open black squares the unchallenged control calf group.

5.3.6 Gene expression following PT32 Stx2c+ challenge

All the genes of interest had a fold change increase in transcript at day 7 (see Figure 58) which generally decreased towards pre-challenge levels by day 26 in both challenged and unchallenged controls. CD19, CD86 and POU2AF1 fold changes in transcript levels were very similar between challenged and unchallenged controls at both day 7 and 26 time points which is reflected by very similar predicted means and overlapping confidence intervals for both treatment groups (Figure 61 in Appendix and Table 18 in Appendix). CCL21, CD80, CXCL13, LTB and SH2D1A showed similar patterns, with a fold change increase at day 7 and then a decrease towards pre-challenge levels by day 26. Generally the challenged group had a greater fold change increase compared to the unchallenged controls. CCR7, CD22, IL2RA and MS4A1 showed a similar pattern with a fold change increase in the controls at day 7 and then a decrease towards pre-challenge levels by day 26. For these transcripts the challenged group followed a similar pattern but at a lower level than the unchallenged control group. There was no statistically significant difference between treatment groups, or any significant treatment × time interaction interactions for any of the genes of interest (see Table 16 in appendix for *p*-values). There was a statistically significant difference in the fold change transcript levels for CD80, CD86, MS4A1, IL2RA and LTB over time which was independent of treatment ($p = 0.004$, $p = 0.001$, $p = 0.032$, $p = 0.018$ and $p = 0.011$ for CD80, CD86, MS4A1, IL2RA and LTB, respectively).

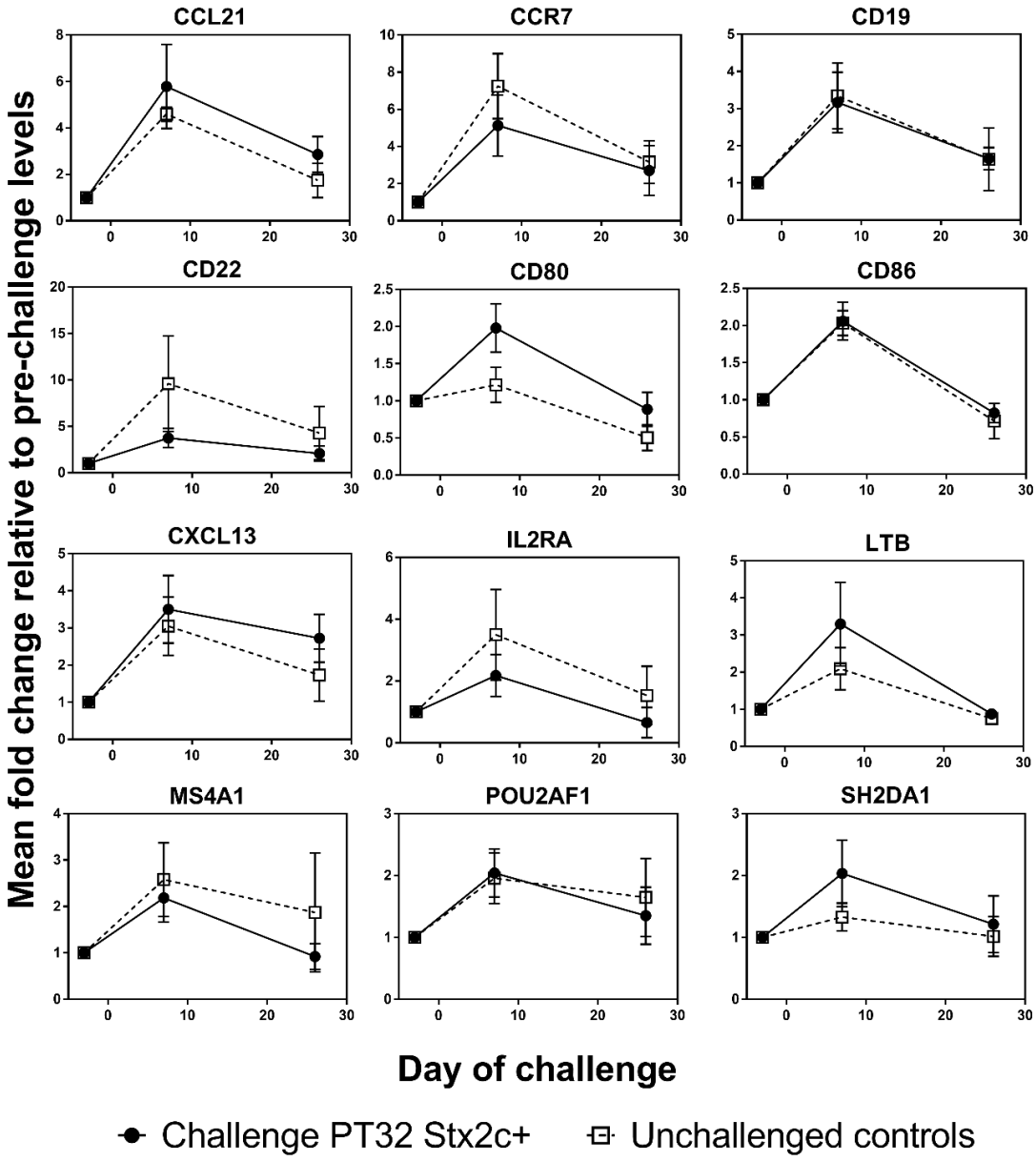


Figure 58: Fold change relative to pre-challenge, gene expression at the terminal rectum of calves orally challenged with STEC O157 PT32 Stx2c+. The graphs represent mean fold changes (± SEM) in gene copy numbers relative to the pre-challenge (day -3) gene copy numbers at day -3, 7 and 26 for each gene considered. The gene copy number is calculated using a plasmid standard for each gene and normalisation to two housekeeping genes. The black circles represent the mean of the challenged calf group and the open black squares the unchallenged control calf group.

5.3.7 Gene expression following PT21/28 Stx2a+Stx2c+ challenge

CCL21, CCR7, CD19, CD22, CD80, CD86, CXCL13, LTB, MS4A1 and POU2AF1 showed a similar pattern in fold change compared to pre-challenge transcript levels for the challenge group increasing at day 7 and then reducing towards pre-challenge levels by day 26 (Figure 59). Fold-changes in the unchallenged control group also showed a general increase at day 7, but these changes were less pronounced compared than those seen in the challenged group. The SH2D1A gene transcripts showed a slightly different pattern with the challenged animals having a further fold change increase at day 26. However there was no statistically significant difference between treatment groups or any treatment × time interactions for any of the above genes (see Table 17 in Appendix for *p*- values). The predicted means for challenged and unchallenged controls were very similar and there was overlapping CI from the linear mixed model (Figure 62 in Appendix and Table 18 in Appendix). CD80, CD86 and LTB did show a statistically significant difference in fold change over time which was independent of treatment group ($p < 0.0001$, $p = 0.0075$ and $p = 0.0261$ respectively).

IL2RA showed a different pattern of gene expression between challenged and unchallenged calves: in the control group there was a fold-change increase in IL2RA transcripts over time, whereas in the challenged group, IL2RA transcripts decreased at day 7 before increasing by day 26 to levels still below those of the unchallenged controls. This was reflected in a statistically significance treatment × time interaction ($p = 0.0194$), although there was no overall difference in the means of the two treatment groups ($p = 0.6727$).

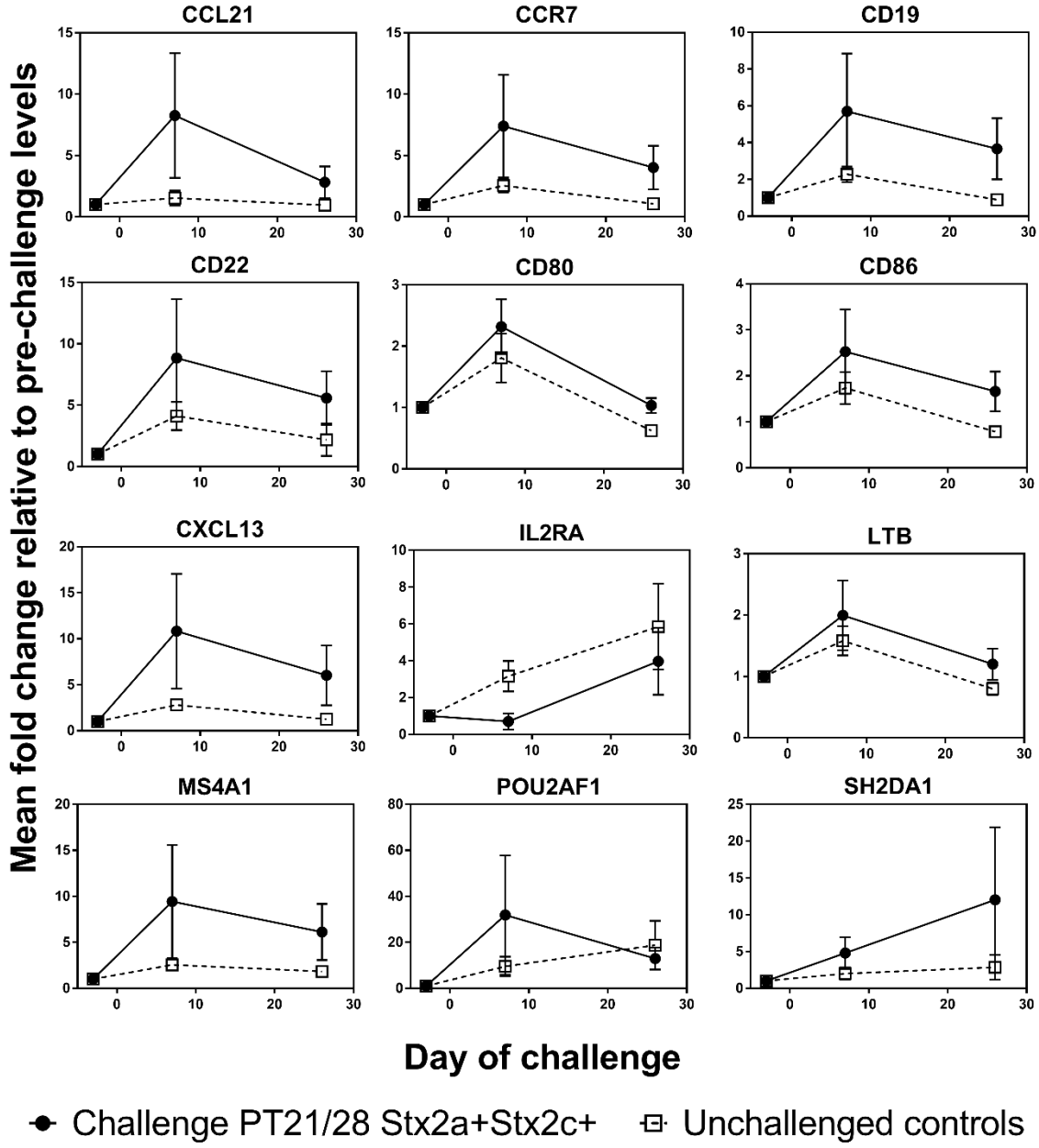


Figure 59: Fold change relative to pre-challenge, gene expression at the terminal rectum of calves orally challenged with STEC O157 PT21/28 Stx2a+Stx2c+. The graphs represent mean fold changes (± SEM) in gene copy numbers relative to the pre-challenge (day -3) gene copy numbers at day -3, 7 and 26 for each gene considered. The gene copy number is calculated using a plasmid standard for each gene and normalisation to two housekeeping genes. The black circles represent the mean of the challenged calf group and the open black squares the unchallenged control calf group.

5.4 Discussion

It has been previously demonstrated that the terminal rectum is the primary site of colonisation for STEC O157 in the bovine host[27]. However STEC O157 can be isolated from multiple sites within the bovine gastro-intestinal tract[277]. The terminal rectum is accessible for taking repeated biopsies with minimal stress to the animal. Such sampling allows the interaction between the bacteria and the mucosal surfaces to be studied at different stages of colonisation. Sampling the same calf repeatedly also allows us to account for inter-animal variation. In this study the bacterial counts indicated that peak shedding for oral challenged calves occurred between 5 and 7 days hence confirming the rationale for sampling at 7 days post challenge and then at post mortem (26 days post challenge) in the orally challenged and unchallenged control calves. A previous study demonstrated the stability of TNF-α gene expression in a similar study involving multiple rectal biopsy taken from control (unchallenged) calves over time, indicating that the process of taking the biopsies does not appear to induce a general inflammatory response within the rectal mucosa[97]. In this study, in order to mitigate the effects of the biopsy procedure on the rectal transcriptome, biopsies were taken at different sites in the rectum at each time point. Furthermore, two biopsies were taken at opposing sites at each time point to try and capture a true representation of the rectal transcriptome, with equal quantities of RNA from each site pooled for down-stream transcriptional analysis.

For the reverse transcriptase quantitative PCR (RT-qPCR) to avoid problems with variation in PCR efficiency which can affect relative quantitative approaches absolute quantification of gene copy number was used, with serial dilutions of a plasmid used to generate a standard curve for each gene on each qPCR plate. The copy number was then normalised to the geometric mean of the most suitable two reference genes, which for the samples used in this study were determined to be ATP5B and GAPDH. The number of cDNA gene copies at the start of each qPCR reaction will depend on the number of mRNA transcripts per unit mass of tissue processed but also on the variable efficiency of each RT reaction, errors in spectrophotometric estimation of RNA concentration and pipetting inaccuracies. Thus to control for this unavoidable technical variation, a suitable reference is required, against which expression of the gene of interest could be compared. Other studies use relative quantification which compares the expression of the gene of interest versus a single reference gene, by comparing the change in the C_T value for each gene between two

experimental conditions[278,279]. The C_T value is the PCR cycle number when the fluorescent signal from the qPCR reaction intersects with a threshold set on the linear part of the amplification curve at the start of the exponential phase. The C_T value is affected by the reaction conditions including the master mix, cycler and efficiency of the primers used. Previous studies have shown that a single reference gene is inappropriate and that at least two carefully selected reference genes should be used for accurate RT-qPCR experiments[276]. Therefore in this study two genes were selected for use as reference genes.

The gene transcripts to be quantified were chosen following a recent study in the literature discussed in the introduction of this chapter[129] and also another two genes, CD80 and CD86, which are involved in the induction of the adaptive immune response. The previous study by Wang *et al*. (2016) had demonstrated down-regulation of these ten genes at the terminal rectum in cattle super shedding STEC O157 compared to cattle sampled negative for STEC O157. In this current study the only statistically significant difference we determined between STEC O157 challenged calves and unchallenged control calves was a reduced mean fold change in transcripts of IL2RA in PT21/28 Stx2a+Stx2+ challenged calves compared to control calves at the time of peak shedding. No other statistically significant differences occurred between challenged and unchallenged control calves with the other genes of interests or STEC O157 challenge strains. The challenge group is generally associated with higher fold changes in gene expression, particularly for the PT2128 Stx2a+Stx2c+ challenge strain, but the control unchallenged group was higher in several cases as well. The overall difference in mean fold change from 7 to 26 days post challenge is statistically significant in several cases, but this is not always in the same direction. There was an overall statistically significant fold change difference from 7 to 26 days post challenge for genes CD80 and CD86 for the three strains, significant differences between days post challenge are obtained for some additional genes for PT32 Stx2c+ challenge strain (IL2RA, LTB, MS4A1) and PT2128 Stx2a+Stx2c+ challenge strain (IL2RA, LTB). For IL2RA in the PT32 Stx2c+ challenge trial the fold change is lower at day 26 compared to day 7, whereas in the PT21/28 Stx2a+Stx2c+ challenge trial the fold change is higher at day 26 compared to day 7.

This current study was using much younger animals of a different breed (Holstein-Friesian) than in the Wang *et al*. (2016) study, which used adult British cross Continental feedlot yearling steers fed on a barley-grain based finishing diet[129]. As discussed in the introduction, age can effect immune gene transcription in the gastrointestinal tract[265,266]; indeed in this study we found that transcript levels often increased over time for a number of genes in the unchallenged control calves, which may be related to ongoing development of the calves'

intestinal immune system and which could have partly confounded our results. Unfortunately due to the nature and size of the containment level 3 facilities at Moredun Research Institute, we were unable to perform these experiments on adult animals in which the mucosal immune system may be more transcriptionally stable.

Another important consideration is that the calves in the present study were experimentally orally challenged with a large bolus dose of STEC O157; in the Wang *et al.* (2016) study cattle were naturally colonised with STEC O157. This oral challenge model is unlike the natural situation in which calves are more likely to be ingesting smaller numbers of bacteria over a more prolonged period. Therefore the resulting immune response may be different, with a large bolus more likely to result in a more coordinated and potentially more robust local immune response compared to that seen in the field.

The cattle in the study by Wang *et al.* (2016) had varying levels of shedding pre-slaughter and at slaughter when the biopsy samples were taken; 2 of the five cattle were actually shedding less than 10^4 CFU/g in their faeces on the day of slaughter[129,280]. Super shedding cattle can be grouped into three different types: persistent shedders which shed for several months, moderate shedders which shed for about 30 days and non-persistent shedders which shed for less than 14 days[281]. Due to the nature of the Wang *et al.* (2016) study it is not known which group the super shedding cattle were in. Because there were only 5 super shedding cattle, this group could have been highly skewed by a number of non-persistent super shedders. There is certainly lots of heterogeneity in natural STEC O157 shedding, with some studies noting significant variation in shedding levels even within a day[282,283].

Challenge strains are also likely to be different in this present study compared to the naturally occurring strains in Canada in the study by Wang *et al.* (2016). In this experimental study defined strains of STEC O157 were used for the challenge, but in the Wang *et al.* (2016) study it is highly likely that multiple strains of STEC O157 along with non-O157 STEC strains were circulating within the feedlot. We do not know if all the super shedders were shedding the same strain and/or if they were shedding multiple strains of STEC O157. A number of studies have shown that individual cattle can shed multiple strains of STEC O157[284-286]. Challenge with different strains of STEC O157 has led to differences in immune responses in our study, e.g. differences between challenges with strains PT21/28 Stx2a+Stx2c+ and PT21/28 Stx2c+ which only differ in the expression of a functional A subunit of Stx2a+. Another study has demonstrated significant difference in host responses to PT32 Stx2c+ and PT21/28 Stx2c+[97]. As discussed in the introduction chapter, not only are

Stx proposed to affect the host immune response, the bacteria produce other effector molecules which are likely to affect the host immune response. The production of these effector molecules will differ between STEC O157 strains, thus any suppressive effect seen with one STEC O157 strain may not be seen with another STEC O157 strain even if they produce the same Stx types.

Wang *et al.* (2017) have now also demonstrated that samples taken at post mortem from the distal jejunum and descending colon of super shedding cattle showed potentially higher levels of transcripts involved in T-cell migration and proliferation. This agrees with Corbishley *et al.* (2014) who found an increased expression in Ki-67 a marker for proliferation on T-cells in the rectal lymph nodes of STEC O157 challenged calves[97]. The combination of these two studies by Wang *et al*. suggest a higher level of T-cell migration in locations anterior to the recto-anal junction, while lower T-cell migration and quantity at the recto-anal junction, which suggests a potential dysregulation of the migration of T-cell in super shedding cattle at the rectal site[129,280]. The 2017 study by Wang *et al*. has also demonstrated transcriptome analyses suggesting inhibition of B-cell signalling pathways in the distal jejunum and the trend of reduction in lymphocyte activation and migration in the cecum of super shedding cattle[280].

A RNA-seq study using a *stx* negative *E. coli* O157 challenge strain, demonstrated that changes in gene expression (between challenged and unchallenged control calves) was remarkably higher in the ileal Peyer's patches (1159 genes) than the RAJ (15 genes) during primary infection[130]. However the effect was much less obvious after re-infection with the *stx* negative *E. coli* O157 challenge strain (17 and 10 genes respectively)[130]. At the RAJ only one gene (FABP2 with encodes for fatty acid binding protein 2, which is not directly involved in immune function) was affected by primary and secondary infection. In the ileal Peyer's patches 7 genes were affected by both primary and secondary infection[130], although as discussed in the introduction, this study was confounded as primary and secondary infected calves were of different ages and weaning status. Our calves were screened pre-challenge 5 times at weekly intervals for STEC O157 shedding, but we still cannot categorically rule out that the calves were not previously exposed to STEC O157 prior to oral challenge. If this was their second exposure to STEC O157 this may have affected the results from the current study.

IL2RA is the only gene with statistically significant differences between the PT21/28 Stx2a+Stx2c+ challenged and unchallenged control calves in this study. IL2RA encodes for

IL-2 receptor alpha chain (also called CD25), which, together with the beta and common gamma chain, constitute the IL-2 receptor[287]. Different associations of the individual subunits can produce 3 different IL-2 receptors which differ in their affinity to IL-2, IL2RA is essential for the high affinity IL-2 receptor[287]. However the IL-2 receptor of intermediate affinity does not require IL2RA[288]. IL2RA is up-regulated on activated T-cells and we have used IL2RA (CD25) as a marker of T-cell activation in chapter 4. IL-2 is produced by activated $CD4^+$ T_H1 cells, and Corbishley *et al.*(2014) demonstrated a T_H1 skew in response to STEC O157 challenge and a reciprocal down-regulation of T_{REG} response[97].

The data from this study, and the previous transcriptomic studies in the literature[129,130,280], demonstrate the complexity of trying to understand the effect of STEC O157 colonisation in cattle. This study demonstrated no change in 11 specific genes related to immune function at the terminal rectum between orally STEC O157 challenged calves and controls. It is difficult to conclude if the different findings by others is because of the difference in study design, i.e. different age of calves, different challenge type or because the cattle in the Wang *et al.* (2016) study became super shedders due to an inherent defect in their immune system at the terminal rectum which allowed the bacteria to colonise and shed at high levels[129]. Further studies involving repeating rectal biopsies from adult cattle during the time-course of natural supper-shedding events would help to clarify this further. It should also be noted that the studies by Wang *et al.* (2016 and 2017) and Kieckens *et al.* (2016) did not demonstrate consistent down-regulation of the same specific genes related to immune function, indicating that significant variation in the immune genes affected by STEC O157 colonisation occurs in different study systems. By restricting this study to only 12 immune response genes, it is likely that many genes that were differentially expressed between challenged and control calves were missed. RNA sequencing analysis of the rectal biopsy samples collected in this study would allow the full extent of any potential immunomodulatory effects of the STEC O157 strains tested to be determined.

Chapter 6
General Discussion

The objective of this thesis was to further characterise humoral and cellular responses in bovine animals colonised with STEC O157, with a focus on how STEC O157 modulate adaptive immune responses in the bovine host. This study started by characterising the antibody responses in cattle in a natural colonisation study and then went on to study how a toxoid vaccination could affect STEC specific antibody responses in a natural colonisation situation. An experimental challenge calf study allowed analysis of both STEC O157 specific antibody responses and cellular responses to STEC O157 in a more controlled experimental setting. The experimental studies enabled comparison of immune responses when calves were orally challenged with a genetically modified STEC O157 strain (PT21/28 Stx2a+Stx2c+) only differing in the *stx2a* A subunit compared to the wild type PT21/28 Stx2c+ strain and also another clinically relevant PT32 Stx2c+ strain. These experimental challenge studies also enabled us to compare the immune responses to a non-STEC O157 antigen OVA (immunisations) in calves challenged with STEC O157 and calves which were not STEC O157 challenged. Allowing us to try and decipher if colonisation with STEC O157 is affecting an animals ability to mount immune responses to concurrent immunisations.

In the natural colonisation chapter there is some evidence to support suppression of the bovine immune system by STEC O157. In animals which were shedding but not super shedding STEC O157 there were lower Tir-specific IgA compared to STEC O157 negative animals. There was also significantly higher Tir-Specific IgA in super shedding animals compared to the STEC O157 negative animals. This supports the hypothesis that STEC O157 could be suppressing antibody responses but not in super shedding animals. One of the main limitations of the natural colonisation studies was the lack of information about the STEC O157 strains which the cattle were shedding. In the USA trial the cattle were only faecal sampled at one time point so although they were grouped into negative (non-shedding), positive or super shedding this grouping was done from only one faecal sample

and data in the literature indicates the heterogeneity of STEC O157 shedding even within one animal[70,282,289]. Thus some of the negative animals may not have been shedding at that specific time point, but could have been shedding hours before or after. Also although more animals were used both in the USA natural colonisation trial and the toxoid vaccination trial compared to the experimental challenge trials there were still relatively low number of animals. This section of the study could be extended to include more cattle on different units, and sampling could including STEC O157 strain typing. This would give us more information on how robust the suppression of antibody responses determined was, and if it was related to the *stx* repertoire of the STEC O157 strain the cattle were shedding.

The toxoid immunisation trial performed in Germany provides evidence that the toxoid vaccination may have potential as a future STEC O157 vaccine. The concept of toxoid vaccinations are of interest as possible vaccines for other toxin producing pathogens. The toxoid vaccination in this study reduced shedding levels in cattle and there was some evidence that the toxoid vaccine was able to inhibit the suppressive effect on the host's humoral immune response by the STEC O157 bacteria. H7, H2 and H21 specific IgG_1 antibody levels were all higher (at week 9-12) in the toxoid vaccinated calves compared to placebo control calves, even though less STEC O157 was detected in the vaccinated calves compared to the control calves. This indicates that vaccination with the toxoid allows cattle to mount an increase H7, H2 and H21 specific antibody response to STEC O157. Although there could be other mechanisms involved as discussed in the discussion of chapter 2, this data does fit in with the hypothesis that Stx produced by STEC O157 are suppressing the bovine immune response.

The toxoid vaccination study did not involve an oral experimental challenge, however it was performed under controlled conditions, in a select group of calves at set ages etc. In the toxoid vaccine study there were a number of circulating STEC O157 strains with different H-types, but we still do not know at this stage how this vaccine would work in a field situation, with different strains of STEC O157 or against other non-O157 STEC. Future trials with larger numbers of animals of different ages, breeds and kept in different management conditions would be necessary to fully conclude the effect of toxoid vaccination on STEC O157 colonisation. A field vaccine trial in more natural challenge conditions would be of interest and whole genome sequencing of any strains shed in the trial would give us more information on how the vaccines works against STEC strains producing different Stxs. An interesting area of work would also be to combine the toxoid vaccination with STEC antigen

subunit vaccines already in development[40,290] to determine if the toxoid vaccination would enhance the efficacy of the subunit vaccines against STEC O157.

The experimental oral challenge studies, aimed to demonstrate the immune responses in a more controlled manner, using STEC O157 strains with different *stx* repertoires. The cellular and humoral response to STEC O157 oral challenge results (chapter 3) are broadly consistent with previous data in the literature. The cellular responses to T3SP by rectal lymph node cells concurs with Corbishley *et al.* (2014) findings, with colonised calves showing cellular responses to the T3SP but also unchallenged calves showed responses. Multiple studies have looked at STEC O157 antigen specific antibody responses following experimental STEC O157 challenge[115-117,126,236] and our data fits in with the often variable and weak antibody responses detected following oral STEC O157 challenge. Interestingly the local STEC O157 antigen specific antibody responses at the terminal rectum, generally were higher in the calves challenged with the PT21/28 Stx2a+Stx2c+ calves compared to the calves challenged with the other two strains (Stx2c+ positive). This did not fit with our general hypothesis that Stx would suppress the immune response. Challenge with a *stx* negative strain would have helped to clarify these results further.

The results from chapter 5, when targeted PCRs were used to compare transcripts for specific immune genes at the rectal anal junction were unexpected and did not consistently agree with the results from the initial study by Wang *et al.* (2016). It was expected with the experimental challenge studies to see suppression of the transcripts for the specific immune genes following STEC O157 challenge as was determined by Wang *et al*. (2016) in the natural challenge situation. However we did not see consistent suppression following STEC O157 challenge. These inconsistent results suggest that due to the complex interactions between bacteria challenge, host responses and possibly gastrointestinal bacteria the results will vary depending on the bacterial strain, challenge type, animal age and possibly other external factors as discussed previously. Performing RNA sequencing on the RNA extracted from the rectal biopsies taken in these trials would give us further information to support or contradict the data in the literature suggesting immune suppression at the terminal rectum in STEC O157 colonised calves. However the calves in this study were experimentally challenged and the antibody and cellular responses both systemically and locally did not consistently suggest host immune response suppression by the STEC O157 challenge strains used in this study. Wang *et al.* (2017) have more recently shown that in the same cattle as the original study[129] there was not consistent suppression of the same immune gene transcripts

throughout the gastrointestinal tract in super shedding cattle compare to the non-shedding control cattle.

Chapter 4 demonstrates that STEC O157 colonisation did not significantly affect the ability of the host animal to respond to a concurrent immunisation under these experimental conditions. This is the first study to our knowledge to examine the effect of experimental STEC O157 challenge on concurrent immunisations. Unexpectedly the OVA-specific cellular and antibody responses were not consistently down regulated in STEC O157 challenged calves compared to their unchallenged control group, in fact there were some enhanced local OVA immune response in calves challenged with the PT21/28 Stx2c+ strain. There was a statistically significant increase in OVA specific $CD4^{+}CD25^{+}$ cells in PT21/28 Stx2c+ challenged and OVA immunised calves compare to immunised only controls. There was also a statistically significant decrease in OVA specific IL-10 in PT21/28 Stx2c+ challenged and OVA immunised calves compared to immunised only controls. One explanation of these findings is that the Stx2a B subunit is acting to enhance the response in these challenged calves, however further work would need to be done to clarify this hypothesis.

The OVA immunisations were given to calves which were experimentally orally challenged with STEC O157 and we were also not able to demonstrate significant consistent immune suppression to the bacteria under these conditions (chapters 3 and 5). A field study with natural challenge and OVA-immunisations may give a different result but this would be more difficult to control, as previous data in the literature suggested that it was the priming of the immune response that is suppressed by Stx and in field conditions timing the primary immunisations with STEC O157 colonisation would be difficult to accurately achieve. The study by Wang *et al.* (2017), have also demonstrated that the STEC O157 in the natural challenge does not suppress immune responses throughout the gastrointestinal tract. They suggest that the suppression only occurs at the rectal anal junction and thus allowing colonisation to occur at this site, where the bacteria is able to exert its suppressive effects[129,280]. The data from the Wang *et al.* (2017) study does not support the hypothesis that STEC O157 colonisation would have a systemic immune suppressive effect.

Some parts of this study are to continue after this PhD is finished including RNA sequencing analysis of the rectal biopsies from chapter 5. As discussed this will enable to look at the abundance of transcripts of other genes that could be affected by STEC O157 challenge. It will also give us the opportunity to further analysis the effect of the repeated biopsy

technique. This study has indicated some differences in antigen specific antibody levels between STEC O157 positive and negative animals; also in chapter 3 between toxoid vaccinated and placebo vaccinated calves. To examine this further, it would be interesting to determine the affinity of these antibodies for the antigens in question, there are laboratory techniques available. This may give a better indication of the potential biological impact of the differences noted. Antibody affinity is recognized as being an important parameter in the immune response in terms of clearance of pathogens and response to vaccination[291]. Adjuvants and synthetic immunomodulators are capable of altering antibody affinity, as well as the actual levels of antibody production[292]. It would be interesting to see if the toxoid vaccination in particular was capable of leading to a change in antibody affinity as well as the level of H7 specific IgG_1 produced.

As already discussed larger field studies determining antibody responses to EHEC antigens and also antibody responses to other concurrent vaccinations would allow us to determine if what has been demonstrated in this thesis is also mirrored in the field situation. However these field experiments are more difficult to control and potentially any subtle differences may be even more difficult to determine due to natural variation in the field unless very large numbers of animals were used. Depending on the results of RNA sequencing which is to be carried out on the rectal biopsies from the experimental challenge, repeated rectal biopsy of naturally STEC O157 challenged cattle over an infection would also be useful.

One of the main limitations of the experimental challenge sections of this study was the lack of challenge with an *E. coli* O157 *stx* negative strain as a direct comparison, to really compare what effect Stx specifically was having on the immune response. Due to the lack of significant results in the trials already performed between challenged and unchallenged calves, ethically and financially a *stx* negative challenge trial was not performed. However we were able to challenge with strains differing only in their *stx2a* A subunit and follow the immune responses, although having an active B subunit may have complicated the results demonstrated. There is some evidence within the group that the Stx activity may be effected by the production of other Stx within the strain so this may also have complicated the results with the PT21/28 Stx2a+Stx2c+ strain challenges (Dr Stephen Fitzgerald, personal communication).

All of the strains used in the oral challenge trials possessed *stx2*, as stated previously these strains were chosen as they are clinically relevant strains in Scotland[30]. Some of the previous studies in the literature indicating Stx has suppressive effects on bovine immune cells have

used Stx1[136,140] rather than Stx2. Stx1 and Stx2 are immunologically distinct and share only 56 % amino acid sequence identity[41]. In humans, strains possessing Stx2 are more likely to lead to severe disease[42] and in mice Stx2 has been shown to be more toxigenic[43]. However we do not really know the different effect Stx1 compared to Stx2 might have on bovine immune cells. This in part could account for some of the differences in the results shown in these trials compared to what was expected from the previous research in the literature. Future experiments comparing the effect of Stx1 and Stx2 on bovine immune cells *in vivo* would be interesting.

The natural colonisation chapter indicated that there was some STEC specific antibody suppression in STEC O157 positive cattle compared to non-shedding controls but in the experimental challenge studies we did not demonstrate consistent immune suppression with STEC O157 oral challenge. Due to the low number of animals in the experimentally challenged calves the calves were not split into super shedding and positive shedding calves. In fact all of the calves challenged in the experimental study were shedding > 10^4 CFU/g faeces at some point over the study period. So they could all have been grouped as super shedders, but actually many of them only super shed for a few days early on in the trial. The suppression on Tir specific IgA was not demonstrated in super shedding cattle in the natural colonisation chapter only cattle shedding > 0 but < 10^4 CFU/g faeces STEC O157.

The OVA study was also performed on the experimental orally challenged calves and again no immune suppression was demonstrated to OVA in these experimental conditions with STEC O157 challenge. The experimental challenges are different to the field (natural colonisation) situation, the calves are challenged with one large bolus of STEC O157, rather than in the field when we assume they will gradually ingest smaller numbers of STEC O157 over a more prolonged period of time. Also due to the nature of the unit, cost and safety we were only able to use young calves in the experimental challenge studies. This again may affect the results, these calves would not have fully developed immune systems, and in in fact they would be developing their immune system for the duration of the trial. We were limited with animal numbers in the experimental challenge studies. There was often large intra group variability and the response from one calve was able to significantly skew the results. Although OVA has been used in previous trials as a model vaccine antigen[207-209] this study found that the immune responses to OVA in unchallenged OVA immunised control calves varied significantly within the group. This may have been due to concurrent infections, age and genotype of the calves or other external factors. A different vaccination regime using three immunisations and following the calves over a longer period of time may

have helped to get a more consistent OVA immune response or using a different adjuvant may have led to a more robust immune response to follow.

A complicating factor with studying STEC O157 in cattle, is the limit of detection of the assays used to determine cattle as STEC O157 negative. Using immune-magnetic separation, where O157 specific antibodies bound to magnetic beads are used following enrichment of faecal samples, the overall sensitivity for detecting STEC O157 in cattle faeces has been estimated to be as low as 56 %; and if shedding is 10^2 CFU/g faeces the sensitivity may be as low as 20 %[293]. It can be unclear if animals shedding at low levels that are difficult to detect (i.e. less than 10^2 CFU/g faeces) are in fact colonised or merely represent flow through of ingested bacteria. It has been suggested that cattle that persistently shed EHEC O157 may do so at low levels[294]. Thus some of the animals in this study which were categorised as negative may not actually be negative for STEC O157, they may be persistent low level shedders or shedding low levels at the beginning or end of STEC O157 colonisation or at least been exposed to STEC O157 and shedding very low levels due to flow through in the faeces. The calves in the oral challenge experimental studies which were classed as negative were sampled multiple times and also PCR was performed on faecal samples pre-trial looking for *stx* and STEC O157 specific genes to try and increase the sensitivity of finding STEC O157 if it was present.

Although this study was looking at STEC O157 experimental challenges, it demonstrates the variation seen between experimental challenges and natural colonisation in cattle which may be relevant to other bacteria challenge studies. As discussed this may be due to the nature of the challenge (i.e. large oral bolus of bacteria) but also the conditions which the calves were kept in and the very limited number of animals in the experimental challenges. These factors need to be considered when planning future experiments especially if only marginal changes in immune responses are expected.

An implication of the findings from the OVA immune response chapter is that it is unlikely that STEC O157 infections in cattle are likely to be affecting their ability to respond to concurrent vaccinations. Any systemic effect on immune response by STEC O157 colonisation seems to be minor. Interestingly our wild type strain did not have an active Stx2a A subunit and actually in this study having an active A subunit did not appear to be an advantage to the bacteria. Strains which do not produce Stx, where the Stx is inactivated due to insertion sequences are isolated in the field again suggesting that Stx may be disadvantageous to the bacteria[295]. Another complicating factor for these experimental

challenge studies is that we do not know how stable the insertion sequence is within the PT21/28 Stx2a+Stx2c+ strain at this stage and what other insertion sequences could be inserting into the bacteria genome and affecting the strains ability to produce toxin, attach, survive etc. within the host animal during these experiments. Long read sequencing performed by the group on PT21/28 Stxa+Stx2c+ bacteria recovered from challenged calves in this study, has identified large chromosomal inversions in the genomes of the challenge strains recovered from shedding animals, suggesting the bacterial genome of the challenge strain is not stable *in vivo*. Whether these inversions were present in the bacterial innocula or occurred within the host is unclear, as are the implications of these inversions on the bacterial phenotype (Fitzgerald *et al.,* in preparation).

If the original hypothesis for the OVA immunisation chapter was correct, then STEC O157-mediated suppression of host immune responses to concurrent vaccination could have been used to encourage the farming industry to use STEC O157 vaccinations. At the moment there is little incentive for farmers to vaccinate their cattle because STEC O157 does not cause clinical disease in cattle. STEC O157 vaccination would still be advantageous, reducing STEC O157 shedding levels in cattle has been predicted to reduce the burden of disease within the human population[5,15]. Future work needs to concentrate on developing an effective STEC O157 control method in cattle, with a vaccination seeming the most likely to be effective. A STEC O157 vaccine needs to be cheap, easy to deliver, reliable and able to protect against multiple strains and serotypes. The toxoid vaccination used in chapter 2 of this thesis, is showing some promising results and using this in combination with other subunit antigens may be an interesting option. Also future work to determine the prevalence of STEC O157 in other different host species that may contribute to the STEC O157 infections in the human population is important and thus evaluating the use of any future STEC O157 vaccine developed in these contributing species.

References:

1. Kaper JB, Nataro JP, Mobley HLT. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology* 2004;2:123-140.

2. Karmali MA. Infection by Verocytotoxin-producing *Escherichia-coli*. *Clinical Microbiology Reviews* 1989;2:15-38.

3. Obrien AD, Holmes RK. Shiga and Shiga-like toxins. *Microbiological Reviews* 1987;51:206-220.

4. Locking ME, O'Brien SJ, Reilly WJ, et al. Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. *Epidemiology and Infection* 2001;127:215-220.

5. Chase-Topping M, Gally D, Low C, et al. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nature Reviews Microbiology* 2008;6:904-912.

6. Riley LW, Remis RS, Helgerson SD, et al. Haemorrhagic colitis associated with a rare *Escherichia-coli* serotype. *New England Journal of Medicine* 1983;308:681-685.

7. Taylor CM, White RHR, Winterborn MH, et al. Hemolytic-Uremic syndrome- Clinical-experience of an outbreak in the West-Midlands. *British Medical Journal* 1986;292:1513-1516.

8. Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 2005;365:1073-1086.

9. Ardissino G, Tel F, Possenti I, et al. Early Volume Expansion and Outcomes of Hemolytic Uremic Syndrome. *Pediatrics* 2016;137:9.

10. Mead PS, Griffin PM. *Escherichia coli* O157 : H7. *Lancet* 1998;352:1207-1212.

11. Health Protection Scotland. VTEC/*E.coli* O157 Action Plan for Scotland, 2013-2017. 2013; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1421976/pdf/immunology00094-0127.pdf. Accessed 3rd September, 2017.

12. Scharff R. Economic Burden from Health Losses Due to Foodborne Illness in the United States. *Journal of Food Protection* 2012;75:123-131.

13. Frenzen PD, Drake A, Angulo FJ, et al. Economic cost of illness due to *Escherichia coli* O157 infections in the United States. *Journal of Food Protection* 2005;68:2623-2630.

14. McPherson M, Kirk MD, Raupach J, et al. Economic Costs of Shiga Toxin-Producing *Escherichia coli* Infection in Australia. *Foodborne Pathogens and Disease* 2011;8:55-62.

15. Matthews L, Reeve R, Gally DL, et al. Predicting the public health benefit of vaccinating cattle against *Escherichia coli* O157. *Proceedings of the National Academy of Sciences of the United States of America* 2013;110:16265-16270.

16. Olsen SJ, Miller G, Breuer T, et al. A waterborne outbreak of *Escherichia coli* O157 : H7 infections and hemolytic uremic syndrome: Implications for rural water systems. *Emerging Infectious Diseases* 2002;8:370-375.

17. Armstrong GL, Hollingsworth J, Morris JG. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiologic Reviews* 1996;18:29-51.

18. Ihekweazu C, Carroll K, Adak B, et al. Large outbreak of verocytotoxin-producing *Escherichia coli* O157 infection in visitors to a petting farm in South East England, 2009. *Epidemiology and Infection* 2012;140:1400-1413.

19. Michel P, Wilson JB, Martin SW, et al. Temporal and geographical distributions of reported cases of *Escherichia coli* O157 : H7 infection in Ontario. *Epidemiology and Infection* 1999;122:193-200.

20. Valcour JE, Michel P, McEwen SA, et al. Associations between indicators of livestock farming intensity and incidence of human Shiga toxin-producing *Escherichia coli* infection. *Emerging Infectious Diseases* 2002;8:252-257.

21. Ogden ID, Hepburn NF, MacRae M, et al. Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Letters in Applied Microbiology* 2002;34:100-104.

22. Heuvelink AE, van den Biggelaar F, de Boer E, et al. Isolation and characterization of verocytotoxin-producing *Escherichia coli* O157 strains from Dutch cattle and sheep. *Journal of Clinical Microbiology* 1998;36:878-882.

23. Bielaszewska M, Janda J, Blahova K, et al. Human *Escherichia coli* O157 : H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology and Infection* 1997;119:299-305.

24. Beutin L, Geier D, Steinruck H, et al. Prevalence and some properties of verotoxin (shiga-like toxin)- producing *Escherichia-coli* in 7 different species of healthy domestic-animals. *Journal of Clinical Microbiology* 1993;31:2483-2488.

25. Garcia A, Fox JG. The rabbit as a new reservoir host of enterohemorrhagic *Escherichia coli*. *Emerging Infectious Diseases* 2003;9:1592-1597.

26. Garcia-Sanchez A, Sanchez S, Rubio R, et al. Presence of Shiga toxin-producing *E-coli* O157 : H7 in a survey of wild artiodactyls. *Veterinary Microbiology* 2007;121:373-377.

27. Naylor SW, Low JC, Besser TE, et al. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157 : H7 in the bovine host. *Infection and Immunity* 2003;71:1505-1512.

28. Matthews L, McKendrick IJ, Ternent H, et al. Super-shedding cattle and the transmission dynamics of *Escherichia coli* O157. *Epidemiology and Infection* 2006;134:131-142.

29. Cobbold RN, Hancock DD, Rice DH, et al. Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157 : H7 and its association with supershedders and excretion dynamics. *Applied and Environmental Microbiology* 2007;73:1563-1568.

30. Chase-Topping ME, McKendrick IJ, Pearce MC, et al. Risk factors for the presence of high-level shedders of *Escherichia coli* O157 on Scottish farms. *Journal of Clinical Microbiology* 2007;45:1594-1603.

31. Besser TE, Richards BL, Rice DH, et al. *Escherichia coli* O157 : H7 infection of calves: infectious dose and direct contact transmission. *Epidemiology and Infection* 2001;127:555-560.

32. Naylor SW, Roe AJ, Nart P, et al. *Escherichia coli* O157 : H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology* 2005;151:2773-2781.

33. Jarvis KG, Kaper JB. Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secretion system. *Infection and Immunity* 1996;64:4826-4829.

34. Crepin VF, Shaw R, Abe CM, et al. Polarity of enteropathogenic *Escherichia coli* EspA filament assembly and protein secretion. *Journal of Bacteriology* 2005;187:2881-2889.

35. Cornick NA, Booher SL, Moon HW. Intimin facilitates colonization by *Escherichia coli* O157 : H7 in adult ruminants. *Infection and Immunity* 2002;70:2704-2707.

36. Wong ARC, Pearson JS, Bright MD, et al. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Molecular Microbiology* 2011;80:1420-1438.

37. McDaniel TK, Jarvis KG, Donnenberg MS, et al. A genetic-locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 1995;92:1664-1668.

38. Elliott SJ, Wainwright LA, McDaniel TK, et al. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* 1998;28:1-4.

39. Potter AA, Klashinsky S, Li YL, et al. Decreased shedding of *Escherichia coli* O157 : H7 by cattle following vaccination with type III secreted proteins. *Vaccine* 2004;22:362-369.

40. McNeilly TN, Mitchell MC, Corbishley A, et al. Optimizing the Protection of Cattle against *Escherichia coli* O157:H7 Colonization through Immunization with Different Combinations of H7 Flagellin, Tir, Intimin-531 or EspA. *Plos One* 2015;10:19.

41. Jackson MP, Newland JW, Holmes RK, et al. Nucleotide-sequence analysis of the structural genes for Shiga-like toxin-1 encoded by bacteriophage-(33J from *Escherichia-coli*. *Microbial Pathogenesis* 1987;2:147-153.

42. Boerlin P, McEwen SA, Boerlin-Petzold F, et al. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *Journal of Clinical Microbiology* 1999;37:497-503.

43. Tesh VL. Induction of apoptosis by Shiga toxins. *Future Microbiology* 2010;5:431-453.

44. Bergan J, Lingelem ABD, Simm R, et al. Shiga toxins. *Toxicon* 2012;60:1085-1107.

45. Karch H, Tarr PI, Blelaszewska M. Enterohaemorrhagic *Escherichia coli* in human medicine. *International Journal of Medical Microbiology* 2005;295:405-418.

46. Dallman TJ, Ashton P. M., Byrne L., Perry N.T., Petrovska L., Ellis R., Allison L., Hanson M., Holmes A., Gunn G. J., Chase-Topping M. E., Woolhouse M. E. J., Grant K. A., Gally D. L., Wain J. and Jenkins C. Applying phylogenomics to understand the emergence of Shiga-toxin-producing *Escherichia coli* O157:H7 strains causing severe human disease in the UK. *Microbial Genomics* 2015;1.

47. Olsnes S, Reisbig R, Eiklid K. Subunit structure of Shigella cyto-toxin. *Journal of Biological Chemistry* 1981;256:8732-8738.

48. Donohuerolfe A, Keusch GT, Edson C, et al. Pathogenesis of shigella diarrhea. 9. Simplified high-yield purification of shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. *Journal of Experimental Medicine* 1984;160:1767-1781.

49. Obrien AD, Newland JW, Miller SF, et al. Shiga-like toxin-converting phages from *Escherichia-coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science* 1984;226:694-696.

50. Nassar FJ, Rahal EA, Sabra A, et al. Effects of Subinhibitory Concentrations of Antimicrobial Agents on *Escherichia coli* O157:H7 Shiga Toxin Release and Role of the SOS Response. *Foodborne Pathogens and Disease* 2013;10:805-812.

51. Kimmitt PT, Harwood CR, Barer MR. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerging Infectious Diseases* 2000;6:458-465.

52. Agger M, Scheutz F, Villumsen S, et al. Antibiotic treatment of verocytotoxin-producing *Escherichia coli* (VTEC) infection: a systematic review and a proposal. *Journal of Antimicrobial Chemotherapy* 2015;70:2440-2446.

53. Lindberg AA, Brown JE, Stromberg N, et al. Identification of the carbohydrate receptor for shiga toxin produced by shigella dysenteriae type-1. *Journal of Biological Chemistry* 1987;262:1779-1785.

54. Endo Y, Tsurugi K, Yutsudo T, et al. Site of action of a vero toxin (VT2) from *Escherichia-coli* O157-H7 and of shiga toxin on eukaryotic ribosomes-RNA N-glycosidase activity of the toxins. *European Journal of Biochemistry* 1988;171:45-50.

55. Boyd B, Lingwood C. Verotoxin receptor glycolipid in human renal tissue. *Nephron* 1989;51:207-210.

56. Pruimboom-Brees IM, Morgan TW, Ackermann MR, et al. Cattle lack vascular receptors for *Escherichia coli* O157 : H7 Shiga toxins. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:10325-10329.

57. Zumbrun SD, Hanson L, Sinclair JF, et al. Human intestinal tissue and cultured colonic cells contain globotriaosylceramide synthase mRNA and the alternate Shiga toxin receptor globotetraosylceramide. *Infect Immun* 2010;78:4488-4499.

58. Hoey DEE, Currie C, Else RW, et al. Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. *Journal of Medical Microbiology* 2002;51:143-149.

59. Cohen A, Madridmarina V, Estrov Z, et al. Expression of glycolipid receptors to shiga-like toxin on human lymphocyte B- A mechanism for the failure of long-lived antibody response to dysenteric disease. *International Immunology* 1990;2:1-8.

60. Menge C, Stamm I, Wuhrer M, et al. Globotriaosylceramide (Gb(3)/CD77) is synthesized and surface expressed by bovine lymphocytes upon activation *in vitro*. *Veterinary Immunology and Immunopathology* 2001;83:19-36.

61. Proulx F, Seidman EG, Karpman D. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. *Pediatric Research* 2001;50:163-171.

62. Menge C, Eisenberg T, Stamm I, et al. Comparison of binding and effects of *Escherichia* coli Shiga toxin 1 on bovine and ovine granulocytes. *Veterinary Immunology and Immunopathology* 2006;113:392-403.

63. Ramegowda B, Tesh VL. Differentiation-associated toxin receptor modulation, cytokine production, and sensitivity to Shiga-like toxins in human monocytes and monocytic cell lines. *Infection and Immunity* 1996;64:1173-1180.

64. Menge C, Loos D, Bridger PS, et al. Bovine macrophages sense *Escherichia coli* Shiga toxin 1. *Innate Immunity* 2015;21:655-664.

65. Itoh K, Tezuka T, Inoue K, et al. Different binding property of verotoxin-1 and verotoxin-2 against their glycolipid receptor, globotriaosylceramide. *Tohoku Journal of Experimental Medicine* 2001;195:237-243.

66. Fraser ME, Fujinaga M, Cherney MM, et al. Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J Biol Chem* 2004;279:27511-27517.

67. Cowden JM MJ, Smith-Palmer A, Locking M, Browning, L HJaBS. Health Protection Scotland and Food Standards Agency (Scotland), Joint Annual Report on Infectious Intestinal Disease Associated With Foodborne Illness in Scotland, 2012 In: Scotland HP, ed. scotland, 2012.

68. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia-coli* O157-H7, other enterohemorrhagic *Escherichia coli*, and the associated haemolytic uremic syndrome. *Epidemiologic Reviews* 1991;13:60-98.

69. Pearce MC, Chase-Topping ME, McKendrick IJ, et al. Temporal and spatial patterns of bovine *Escherichia coli* O157 prevalence and comparison of temporal changes in the patterns of phage types associated with bovine shedding and human *E. coli* O157 cases in Scotland between 1998-2000 and 2002-2004. *Bmc Microbiology* 2009;9:13.

70. Matthews L, Low JC, Gally DL, et al. Heterogeneous shedding of *Escherichia coli* O157 in cattle and its implications for control. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:547-552.

71. Omisakin F, MacRae M, Ogden ID, et al. Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *Applied and Environmental Microbiology* 2003;69:2444-2447.

72. Lynn RM, O'Brien SJ, Taylor CM, et al. Childhood hemolytic uremic syndrome, United Kingdom and Ireland. *Emerging Infectious Diseases* 2005;11:590-596.

73. Halliday JEB, Chase-Topping ME, Pearce MC, et al. Herd-level risk factors associated with the presence of Phage type 21/28 *E-coli* O157 on Scottish cattle farms. *Bmc Microbiology* 2006;6:5.

74. Xu XF, McAteer SP, Tree JJ, et al. Lysogeny with Shiga Toxin 2-Encoding Bacteriophages Represses Type III Secretion in Enterohemorrhagic *Escherichia coli*. *Plos Pathogens* 2012;8.

75. Sakuma M, Urashima M, Okabe N. Verocytotoxin-producing *Escherichia coli*, Japan, 1999-2004. *Emerg Infect Dis* 2006;12:323-325.

76. Islam MZ, Musekiwa A, Islam K, et al. Regional Variation in the Prevalence of *E. coli* O157 in Cattle: A Meta-Analysis and Meta-Regression. *Plos One* 2014;9:15.

77. Scheutz F, Nielsen EM, Frimodt-Moller J, et al. Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. *Euro Surveill* 2011;16.

78. Health Protection Scotland. Clinical surveillance of haemolytic uraemic surveillance in Scotland, 2010 emergence of highly virulent *Escherichia coli* O26. 2011. Accessed 18th Febuary 2017, 2017.

79. Chase-Topping ME, Rosser T, Allison LJ, et al. Pathogenic Potential to Humans of Bovine *Escherichia coli* O26, Scotland. *Emerging Infectious Diseases* 2012;18:439-448.

80. Mellmann A, Harmsen D, Cummings CA, et al. Prospective Genomic Characterization of the German Enterohemorrhagic *Escherichia coli* O104:H4 Outbreak by Rapid Next Generation Sequencing Technology. *Plos One* 2011;6:9.

81. Centers for disease control and prevention. *E.coli (Escherichia coli)*. 2015. Accessed 20th May, 2017.

82. Brooks JT, Sowers EG, Wells JG, et al. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J Infect Dis* 2005;192:1422-1429.

83. European Centre for Disease Prevention and Control. Annual Epidemiological Report 2016 – Shigatoxin/verocytotoxin-producing *Escherichia coli* infection. 2016; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC101655/pdf/ii003830.pdf. Accessed 5th May, 2017.

84. Humphrey T, O'Brien S, Madsen M. Campylobacters as zoonotic pathogens: A food production perspective. *International Journal of Food Microbiology* 2007;117:237-257.

85. Arthur TM, Brichta-Harhay DM, Bosilevac JM, et al. Super shedding of *Escherichia coli* O157:H7 by cattle and the impact on beef carcass contamination. *Meat Science* 2010;86:32-37.

86. Elder RO, Keen JE, Wittum TE, et al. Intervention to reduce fecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in naturally infected cattle using neomycin sulfate. *Journal of Dairy Science* 2002;85:151.

87. Zhao T, Doyle MP, Harmon BG, et al. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157 : H7 in cattle by inoculation with probiotic bacteria. *Journal of Clinical Microbiology* 1998;36:641-647.

88. CHR Hansen. Pathogen Reduction. 2017. Accessed 6th May, 2017.

89. Diez-Gonzalez F, Callaway TR, Kizoulis MG, et al. Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 1998;281:1666-1668.

90. Dargatz DA, Wells SJ, Thomas LA, et al. Factors associated with the presence of *Escherichia coli* O157 in feces of feedlot cattle. *Journal of Food Protection* 1997;60:466-470.

91. Berg J, McAllister T, Bach S, et al. *Escherichia coli* O157 : H7 excretion by commercial feedlot cattle fed either barley- or corn-based finishing diets. *Journal of Food Protection* 2004;67:666-671.

92. Magnuson BA, Davis M, Hubele S, et al. Ruminant gastrointestinal cell proliferation and clearance of *Escherichia coli* O157 : H7. *Infection and Immunity* 2000;68:3808-3814.

93. Jacob ME, Parsons GL, Shelor MK, et al. Feeding supplemental dried distiller's grains increases faecal shedding of *Escherichia coli* O157 in experimentally inoculated calves. *Zoonoses and Public Health* 2008;55:125-132.

94. Passport Food Safety Solutions. Finalyse®: A Novel Pre-harvest Hide Wash. 2017. Accessed 10th May, 2017.

95. Stevens MP, van Diemen PM, Dziva F, et al. Options for the control of enterohaemorrhagic *Escherichia coli* in ruminants. *Microbiology-Sgm* 2002;148:3767-3778.

96. Snedeker KG, Campbell M, Sargeant JM. A Systematic Review of Vaccinations to Reduce the Shedding of *Escherichia coli* O157 in the Faeces of Domestic Ruminants. *Zoonoses and Public Health* 2012;59:126-138.

97. Corbishley A, Ahmad NI, Hughes K, et al. Strain-Dependent Cellular Immune Responses in Cattle following *Escherichia coli* O157:H7 Colonization. *Infection and immunity* 2014;82:5117-5131.

98. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. *Nature Reviews Immunology* 2011;11:375-388.

99. Van Donkersgoed J, Hancock D, Rogan D, et al. *Escherichia coli* O157 : H7 vaccine field trial in 9 feedlots in Alberta and Saskatchewan. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* 2005;46:724-728.

100. Thomson DU, Loneragan GH, Thornton AB, et al. Use of a Siderophore Receptor and Porin Proteins-Based Vaccine to Control the Burden of *Escherichia coli* O157:H7 in Feedlot Cattle. *Foodborne Pathogens and Disease* 2009;6:871-877.

101. Thornton AB, Thomson DU, Loneragan GH, et al. Effects of a Siderophore Receptor and Porin Proteins-Based Vaccination on Fecal Shedding of *Escherichia coli* O157:H7 in Experimentally Inoculated Cattle. *Journal of Food Protection* 2009;72:866-869.

102. Macleod DL, Gyles CL. Immunization of pigs with a purified shiga-like toxin-II variant toxoid. *Veterinary Microbiology* 1991;29:309-318.

103. Kerner K, Bridger PS, Kopf G, et al. Evaluation of biological safety i*n vitro* and immunogenicity *in vivo* of recombinant *Escherichia coli* Shiga toxoids as candidate vaccines in cattle. *Veterinary Research* 2015;46:14.

104. Paddock ZD, Renter DG, Cull CA, et al. *Escherichia coli* O26 in feedlot cattle: fecal prevalence, isolation, characterization, and effects of an *E. coli* O157 vaccine and a direct-fed microbial. *Foodborne Pathog Dis* 2014;11:186-193.

105. Cull CA, Renter DG, Bello NM, et al. Performance and carcass characteristics of commercial feedlot cattle from a study of vaccine and direct-fed microbial effects on *Escherichia coli* O157:H7 fecal shedding. *Journal of Animal Science* 2015;93:3144-3151.

106. Toma L, Low JC, Ahmadi BV, et al. An analysis of cattle farmers' perceptions of drivers and barriers to on-farm control of *Escherichia coli* O157. *Epidemiology and Infection* 2015;143:2355-2366.

107. Moxley RA, Acuff GR. Peri- and Postharvest Factors in the Control of Shiga Toxin-Producing *Escherichia coli* in Beef. *Microbiol Spectr* 2014;2.

108. Arthur TM, Bosilevac JM, Brichta-Harhay DM, et al. Transportation and lairage environment effects on prevalence, numbers, and diversity of *Escherichia coli* O157 : H7 on hides and carcasses of beef cattle at processing. *Journal of Food Protection* 2007;70:280-286.

109. Barkocy-Gallagher GA, Arthur TM, Rivera-Betancourt M, et al. Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157 : H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *Journal of Food Protection* 2003;66:1978-1986.

110. Arthur TM, Bosilevac JM, Nou XW, et al. *Escherichia coli* O157 prevalence and enumeration of aerobic bacteria, Enterobacteriaceae, and *Escherichia coli* O157 at various steps in commercial beef processing plants. *Journal of Food Protection* 2004;67:658-665.

111. Elder RO, Keen JE, Siragusa GR, et al. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:2999-3003.

112. Food Standard Agency. E.coli O157: control of cross-contamination. 2014. Accessed 5th May, 2017.

113. Food Standard Agency. Cooking burgers at home. 2017. Accessed 5th May, 2017.

114. Nart P, Naylor SW, Huntley JF, et al. Responses of Cattle to Gastrointestinal Colonization by *Escherichia coli* O157:H7. *Infection and Immunity* 2008;76:5366-5372.

115. Bretschneider G, Berberov EM, Moxley RA. Isotype-specific antibody responses against *Escherichia coli* O157 : H7 locus of enterocyte effacement proteins in adult beef cattle following experimental infection. *Veterinary Immunology and Immunopathology* 2007;118:229-238.

116. Johnson RP, Cray WC, Johnson ST. Serum antibody responses of cattle following experimental infection with *Escherichia coli* O157:H7. *Infection and Immunity* 1996;64:1879-1883.

117. Wray C, McLaren IM, Randall LP, et al. Natural and experimental infection of normal cattle with *Escherichia coli* O157. *Veterinary Record* 2000;147:65-68.

118. Pirro F, Wieler LH, Failing K, et al. Neutralizing antibodies against shiga-like toxins from *Escherichia-coli* in colostra and sera of cattle. *Veterinary microbiology* 1995;43:131-141.

119. Cristancho L, Johnson RP, McEwen SA, et al. *Escherichia coli* O157 : H7 and other Shiga toxin-producing E-coli in white veal calves. *Veterinary Microbiology* 2008;126:200-209.

120. Widiasih DA, Matsuda I, Omoe K, et al. Passive transfer of antibodies to Shiga toxin-producing *Escherichia coli* O26, O111 and O157 antigens in neonatal calves by feeding colostrum. *J Vet Med Sci* 2004;66:213-215.

121. Bretschneider G, Berberov EM, Moxley RA. Enteric mucosal antibodies to *Escherichia coli* O157 : H7 in adult cattle. *Veterinary Record* 2008;163:218-219.

122. Shen XZ, Lagergard T, Yang YH, et al. Systemic and mucosal immune responses in mice after mucosal immunization with group B streptococcus type III capsular polysaccharide-cholera term B subunit conjugate vaccine. *Infection and Immunity* 2000;68:5749-5755.

123. Externest D, Meckelein B, Schmidt MA, et al. Correlations between antibody immune responses at different mucosal effector sites are controlled by antigen type and dosage. *Infection and Immunity* 2000;68:3830-3839.

124. McNeilly TN, Naylor SW, Mitchell MC, et al. Simple methods for measurement of bovine mucosal antibody responses *in vivo*. *Veterinary Immunology and Immunopathology* 2007;118:160-167.

125. Sinski E, Bairden K, Duncan JL, et al. Local and plasma antibody-responses to the parasitic larval stages of the abomasal nematode *Ostertagia-circumcincta*. *Veterinary Parasitology* 1995;59:107-118.

126. Hoffman MA, Menge C, Casey TA, et al. Bovine immune response to Shiga-toxigenic *Escherichia coli* O157 : H7. *Clinical and Vaccine Immunology* 2006;13:1322-1327.

127. Vande Walle K, De Zutter L, Cox E. Oral infection with a Shiga toxin-negative *Escherichia coli* O157:H7 strain elicits humoral and cellular responses but does not protect sheep from colonisation with the homologous strain. *Veterinary Microbiology* 2011;148:317-322.

128. Joris MA, Verstraete K, De Reu K, et al. Longitudinal follow-up of the persistence and dissemination of EHEC on cattle farms in Belgium. *Foodborne Pathog Dis* 2013;10:295-301.

129. Wang O, Liang GX, McAllister TA, et al. Comparative Transcriptomic Analysis of Rectal Tissue from Beef Steers Revealed Reduced Host Immunity in *Escherichia coli* O157:H7 Super-Shedders. *Plos One* 2016;11:19.

130. Kieckens E, Rybarczyk J, Li RW, et al. Potential immunosuppressive effects of *Escherichia coli* O157:H7 experimental infection on the bovine host. *BMC Genomics* 2016;17:1049.

131. Ferens WA, Cobbold R, Hovde CJ. Intestinal Shiga toxin-producing *Escherichia coli* bacteria mitigate bovine leukemia virus infection in experimentally infected sheep. *Infection and Immunity* 2006;74:2906-2916.

132. Fulton BE, Portella M, Radke K. Dissemination of bovine leukemia virus-infected cells from a newly infected sheep lymph node. *Journal of Virology* 2006;80:7873-7884.

133. McNeilly TN, Nisbet AJ. Immune modulation by helminth parasites of ruminants: implications for vaccine development and host immune competence. *Parasite (Paris, France)* 2014;21:51.

134. Obrig TG, Louise CB, Lingwood CA, et al. Endothelial heterogeneity in shiga toxin receptors and responses. *Journal of Biological Chemistry* 1993;268:15484-15488.

135. Brigotti M, Carnicelli D, Arfilli V, et al. Identification of TLR4 as the Receptor That Recognizes Shiga Toxins in Human Neutrophils. *Journal of Immunology* 2013;191:4748-4758.

136. Menge C, Wieler LH, Schlapp T, et al. Shiga toxin 1 from *Escherichia coli* blocks activation and proliferation of bovine lymphocyte subpopulations *in vitro*. *Infection and Immunity* 1999;67:2209-2217.

137. Menge C, Blessenohl M, Eisenberg T, et al. Bovine ileal intraepithelial lymphocytes represent target cells for Shiga toxin 1 from *Escherichia coli*. *Infection and Immunity* 2004;72:1896-1905.

138. Paton JC, Paton AW. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews* 1998;11:450-+.

139. Stamm I, Wuhrer M, Geyer R, et al. Bovine lymphocytes express functional receptors for *Escherichia coli* Shiga toxin 1. *Microbial Pathogenesis* 2002;33:251-264.

140. Menge C, Stamm I, van Diemen PM, et al. Phenotypic and functional characterization of intraepithelial lymphocytes in a bovine ligated intestinal loop model of enterohaemorrhagic *Escherichia coli* infection. *Journal of Medical Microbiology* 2004;53:573-579.

141. Kim DW, Lenzen G, Page AL, et al. The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:14046-14051.

142. Nadler C, Baruch K, Kobi S, et al. The Type III Secretion Effector NleE Inhibits NF-kappa B Activation. *Plos Pathogens* 2010;6:11.

143. Yen HL, Ooka T, Iguchi A, et al. NleC, a Type III Secretion Protease, Compromises NF-kappa B Activation by Targeting p65/RelA. *Plos Pathogens* 2010;6:13.

144. Marches O, Covarelli V, Dahan S, et al. EspJ of enteropathogenic and enterohaemorrhagic *Escherichia coli* inhibits opsono-phagocytosis. *Cellular Microbiology* 2008;10:1104-1115.

145. Zhang J, Sun Z, Sun PP, et al. Microalgal carotenoids: beneficial effects and potential in human health. *Food & Function* 2014;5:413-425.

146. Cassady-Cain RL, Blackburn EA, Alsarraf H, et al. Biophysical Characterization and Activity of Lymphostatin, a Multifunctional Virulence Factor of Attaching and Effacing *Escherichia coli*. *Journal of Biological Chemistry* 2016;291:5803-5816.

147. Cassady-Cain RL, Blackburn EA, Bell CR, et al. Inhibition of Antigen-Specific and Nonspecific Stimulation of Bovine T and B Cells by Lymphostatin from Attaching and Effacing *Escherichia coli*. *Infect Immun* 2017;85.

148. Michelacci V, Grande L, Tozzoli R, et al. Identification of two allelic variants of toxB gene and investigation of their distribution among Verocytotoxin-producing *Escherichia coli*. *International Journal of Medical Microbiology* 2014;304:730-734.

149. Tozzoli R, Caprioli A, Morabito S. Detection of toxB, a plasmid virulence gene of *Escherichia coli* O157, in enterohemorrhagic and enteropathogenic *E. coli*. *Journal of Clinical Microbiology* 2005;43:4052-4056.

150. Abu-Median AB, van Diemen PM, Dziva F, et al. Functional analysis of lymphostatin homologues in enterohaemorrhagic *Escherichia coli*. *Fems Microbiology Letters* 2006;258:43-49.

151. Babb JL, Kiyono H, Michalek SM, et al. LPS regulation of the immune-response-suppression of immune responses to orally administered T0independent antigen. *Journal of Immunology* 1981;127:1052-1057.

152. Asper DJ, Sekirov I, Finlay BB, et al. Cross reactivity of enterohemorrhagic *Escherichia coli* O157 : H7-specific sera with non-O157 serotypes. *Vaccine* 2007;25:8262-8269.

153. Stamm I, Mohr M, Bridger PS, et al. Epithelial and Mesenchymal Cells in the Bovine Colonic Mucosa Differ in Their Responsiveness to *Escherichia coli* Shiga Toxin 1. *Infection and Immunity* 2008;76:5381-5391.

154. Robinson CM, Sinclair JF, Smith MJ, et al. Shiga toxin of enterohemorrhagic *Escherichia coli* type O157 : H7 promotes intestinal colonization. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:9667-9672.

155. Hamm K, Barth SA, Stalb S, et al. Experimental Infection of Calves with *Escherichia coli* O104:H4 outbreak strain. *Scientific Reports* 2016;6:10.

156. Chart H, Smith HR, La Ragione RM, et al. An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5 alpha and EQ1. *Journal of Applied Microbiology* 2000;89:1048-1058.

157. Muller D, Greune L, Heusipp G, et al. Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. *Applied and Environmental Microbiology* 2007;73:3380-3390.

158. Geue L, Segura-Alvarez M, Conraths FJ, et al. A long-term study on the prevalence of shiga toxin-producing *Escherichia coli* (STEC) on four German cattle farms. *Epidemiology and Infection* 2002;129:173-185.

159. Fields PI, Blom K, Hughes HJ, et al. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E-coli* O157:H7 and O157:NM. *Journal of Clinical Microbiology* 1997;35:1066-1070.

160. Frohlich J, Baljer G, Menge C. Maternally and Naturally Acquired Antibodies to Shiga Toxins in a Cohort of Calves Shedding Shiga-Toxigenic *Escherichia coli*. *Applied and Environmental Microbiology* 2009;75:3695-3704.

161. McNeilly TN, Mitchell MC, Nisbet AJ, et al. IgA and IgG antibody responses following systemic immunization of cattle with native H7 flagellin differ in epitope recognition and capacity to neutralise TLR5 signalling. *Vaccine* 2010;28:1412-1421.

162. Waterhouse AM, Procter JB, Martin DMA, et al. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 2009;25:1189-1191.

163. Moussay E, Stamm I, Taubert A, et al. *Escherichia coli* Shiga toxin 1 enhances il-4 transcripts in bovine ileal intraepithelial lymphocytes. *Veterinary Immunology and Immunopathology* 2006;113:367-382.

164. De Cisneros JPJ, Matthews L, Mair C, et al. The transfer of IgA from mucus to plasma and the implications for diagnosis and control of nematode infections. *Parasitology* 2014;141:875-879.

165. Joris MA, Vanrompay D, Verstraete K, et al. Use of Antibody Responses against Locus of Enterocyte Effacement (LEE)-Encoded Antigens To Monitor Enterohemorrhagic *Escherichia coli* Infections on Cattle Farms. *Applied and Environmental Microbiology* 2013;79:3677-3683.

166. Bennett KM, Gorham RD, Gusti V, et al. Hybrid flagellin as a T cell independent vaccine scaffold. *Bmc Biotechnology* 2015;15:12.

167. Coffman RL, Sher A, Seder RA. Vaccine Adjuvants: Putting Innate Immunity to Work. *Immunity* 2010;33:492-503.

168. Renshaw BR, Fanslow WC, Armitage RJ, et al. HUMORAL IMMUNE-RESPONSES IN CD40 LIGAND-DEFICIENT MICE. *Journal of Experimental Medicine* 1994;180:1889-1900.

169. Stavnezer J. Immunoglobulin class switching. *Current Opinion in Immunology* 1996;8:199-205.

170. Wang L, Rothemund D, Curd H, et al. Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. *Journal of Bacteriology* 2003;185:2936-2943.

171. Winstanley C, Morgan JAW. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. *Microbiology-Sgm* 1997;143:3071-3084.

172. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001;410:1099-1103.

173. Shaaban S. CLA, McAteer S., Jenkins C., Dallman T. J., Bono J. L. and Gally D. L. Evolution of a zoonotic pathogen: investigating prophage diversity in enterohaemorrhagic *Escherichia coli* O157 by long-read sequencing. *Microbial Genomics* 2016;2.

174. Corbishley A. Cellular immune responses of cattle to *Escherichia coli* O157:H7: The University of edinburgh, 2015.

175. Asper DJ, Karmali MA, Townsend H, et al. Serological Response of Shiga Toxin-Producing *Escherichia coli* Type III Secreted Proteins in Sera from Vaccinated Rabbits, Naturally Infected Cattle, and Humans. *Clinical and Vaccine Immunology* 2011;18:1052-1057.

176. Balic A, Bowles VM, Liu YS, et al. Local immune responses in sensitized sheep following challenge infection with *Teladorsagia circumcincta*. *Parasite Immunology* 2003;25:375-381.

177. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration-The multistep paradigm. *Cell* 1994;76:301-314.

178. von Andrian UH, Mackay CR. Advances in immunology: T-cell function and migration - Two sides of the same coin. *New England Journal of Medicine* 2000;343:1020-1033.

179. Corbishley A, Connelley TK, Wolfson EB, et al. Identification of epitopes recognised by mucosal CD4(+) T-cell populations from cattle experimentally colonised with *Escherichia coli* O157:H7. *Veterinary Research* 2016;47:11.

180. Butler JE. Bovine immunoglobulins - an augmented review. *Veterinary Immunology and Immunopathology* 1983;4:43-152.

181. Martorelli L, Hovde CJ, Vilte DA, et al. Impact of Infection Dose and Previous Serum Antibodies against the Locus of Enterocyte Effacement Proteins on *Escherichia coli* O157:H7 Shedding in Calves following Experimental Infection. *Biomed Research International* 2015:8.

182. Mestecky J. The common mucosal immune-system and current strategies for induction of immune-responses in external secretions. *Journal of Clinical Immunology* 1987;7:265-276.

183. Bowles VM, Brandon MR, Meeusen E. Characterization of local antibody-responses to the gastrointestinal parasite *Haemonchus-contortus*. *Immunology* 1995;84:669-674.

184. McNeilly TN, Naylor SW, Mahajan A, et al. *Escherichia coli* O157 : H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. *Infection and Immunity* 2008;76:2594-2602.

185. Forrest BD, Labrooy JT, Beyer L, et al. The human humoral-immune-response to *Salmonella-Typhi* TY21A. *Journal of Infectious Diseases* 1991;163:336-345.

186. Fuller CA, Pellino CA, Flagler MJ, et al. Shiga Toxin Subtypes Display Dramatic Differences in Potency. *Infection and Immunity* 2011;79:1329-1337.

187. Sinclair JF, O'Brien AD. Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157 : H7. *Journal of Biological Chemistry* 2002;277:2876-2885.

188. Ridpath JF. Immunology of BVDV vaccines. *Biologicals* 2013;41:14-19.

189. Monney T, Hemphill A. Vaccines against neosporosis: What can we learn from the past studies? *Experimental Parasitology* 2014;140:52-70.

190. Naiman BM, Alt D, Bolin CA, et al. Protective killed Leptospira borgpetersenii vaccine induces potent Th1 immunity comprising responses by CD4 and gamma delta T lymphocytes. *Infection and Immunity* 2001;69:7550-7558.

191. Windeyer MC, Leslie KE, Godden SM, et al. The effects of viral vaccination of dairy heifer calves on the incidence of respiratory disease, mortality, and growth. *J Dairy Sci* 2012;95:6731-6739.

192. Packianathan R, Clough WJ, Hodge A, et al. Prevention of fetal infection in heifers challenged with bovine viral diarrhoea virus type 1a by vaccination with a type 1c or type 1a vaccine. *New Zealand Veterinary Journal* 2017;65:134-139.

193. Glass EJ. Genetic variation and responses to vaccines. *Anim Health Res Rev* 2004;5:197-208.

194. Hewitson JP, Grainger JR, Maizels RM. Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. *Molecular and Biochemical Parasitology* 2009;167:1-11.

195. McSorley HJ, Maizels RM. Helminth Infections and Host Immune Regulation. *Clinical Microbiology Reviews* 2012;25:585-608.

196. Moreau E, Chauvin A. Immunity against Helminths: Interactions with the Host and the Intercurrent Infections. *Journal of Biomedicine and Biotechnology* 2010:9.

197. Urban JF, Steenhard NR, Solano-Aguilar GI, et al. Infection with parasitic nematodes confounds vaccination efficacy. *Veterinary Parasitology* 2007;148:14-20.

198. Borkow G, Bentwich Z. Chronic parasite infections cause immune changes that could affect successful vaccination. *Trends in Parasitology* 2008;24:243-245.

199. Martins ML, Shoemaker CA, Xu DH, et al. Effect of parasitism on vaccine efficacy against *Streptococcus iniae* in Nile tilapia. *Aquaculture* 2011;314:18-23.

200. Steenhard NR, Jungersen G, Kokotovic B, et al. Ascaris suum infection negatively affects the response to a *Mycoplasma hyopneumoniae* vaccination and subsequent challenge infection in pigs. *Vaccine* 2009;27:5161-5169.

201. Krump L, Hamilton CM, Sekiya M, et al. The effect of *Fasciola hepatica* infection on respiratory vaccine responsiveness in calves. *Veterinary Parasitology* 2014;201:31-39.

202. Schutz JS, Carroll JA, Gasbarre LC, et al. Effects of gastrointestinal parasites on parasite burden, rectal temperature, and antibody titer responses to vaccination and infectious bovine rhinotracheitis virus challenge. *Journal of Animal Science* 2012;90:1948-1954.

203. Pleidrup J, Dalgaard TS, Norup LR, et al. *Ascaridia galli* infection influences the development of both humoral and cell-mediated immunity after Newcastle Disease vaccination in chickens. *Vaccine* 2014;32:383-392.

204. Mooney JP, Lee SJ, Lokken KL, et al. Transient Loss of Protection Afforded by a Live Attenuated Non-typhoidal Salmonella Vaccine in Mice Co-infected with Malaria. *PLoS Negl Trop Dis* 2015;9:e0004027.

205. Taniuchi M, Platts-Mills JA, Begum S, et al. Impact of enterovirus and other enteric pathogens on oral polio and rotavirus vaccine performance in Bangladeshi infants. *Vaccine* 2016;34:3068-3075.

206. Menge C, Stamm I, Blessenohl M, et al. Verotoxin 1 from *Escherichia coli* affects Gb(3)/CD77(+) bovine lymphocytes independent of interleukin-2, tumor necrosis factor-alpha, and interferon-alpha. *Experimental Biology and Medicine* 2003;228:377-386.

207. Lutje V, Black SJ. Cellular interactions regulating the *in vitro* response of bovine lymphocytes to ovalbumin. *Veterinary Immunology and Immunopathology* 1991;28:275-288.

208. Foote MR, Nonnecke BJ, Beitz DC, et al. Antigen-specific B-cell responses by neonatal calves after early vaccination. *Journal of Dairy Science* 2007;90:5208-5217.

209. Nonnecke BJ, Waters WR, Goff JP, et al. Adaptive immunity in the colostrum-deprived calf: Response to early vaccination with *Mycobacterium bovis* strain *bacille Calmette Guerin* and ovalbumin. *Journal of Dairy Science* 2012;95:221-239.

210. Mac Sharry J, Shalaby KH, Marchica C, et al. Concomitant Exposure to Ovalbumin and Endotoxin Augments Airway Inflammation but Not Airway Hyperresponsiveness in a Murine Model of Asthma. *Plos One* 2014;9:12.

211. Singh M, O'Hagan DT. Recent advances in veterinary vaccine adjuvants. *International Journal for Parasitology* 2003;33:469-478.

212. Dalsgaard K. Adjuvants. *Vet Immunol Immunopathol* 1987;17:145-152.

213. Ronnberg B, Fekadu M, Morein B. Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix. *Vaccine* 1995;13:1375-1382.

214. Petrovsky N, Aguilar JC. Vaccine adjuvants: Current state and future trends. *Immunology and Cell Biology* 2004;82:488-496.

215. Naessens J, Scheerlinck JP, De Buysscher EV, et al. Effective *in vivo* depletion of T cell subpopulations and loss of memory cells in cattle using mouse monoclonal antibodies. *Veterinary Immunology and Immunopathology* 1998;64:219-234.

216. Baldwin CL, Teale AJ, Naessens JG, et al. Characterization of a subset of bovine lymphocyte-T that express BOT4 by monoclonal-antibodies and function- similarity to lymphocytes defined by human T4 and murine L3T4. *Journal of Immunology* 1986;136:4385-4391.

217. Husband AJ, Brandon MR, Lascelle.Ak. Absorption and endogenous production of immunoglobulins in calves. *Australian Journal of Experimental Biology and Medical Science* 1972;50:491-&.

218. Maloy KJ, Donachie AM, Mowat AM. Induction of Th1 and Th2 CD4(+) T-cell responses by oral or parenteral immunization with Iscoms. *European Journal of Immunology* 1995;25:2835-2841.

219. Valensi JPM, Carlson JR, Vannest GA. Systemic cytokine profiles in BALB/C mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants *Journal of Immunology* 1994;153:4029-4039.

220. Lefevre EA, Carr BV, Prentice H, et al. A quantitative assessment of primary and secondary immune responses in cattle using a B cell ELISPOT assay. *Veterinary Research* 2009;40.

221. Couper KN, Blount DG, Riley EM. IL-10: The master regulator of immunity to infection. *Journal of Immunology* 2008;180:5771-5777.

222. Diaz-San Segundo F, Rodriguez-Calvo T, de Avila A, et al. Immunosuppression during Acute Infection with Foot-and-Mouth Disease Virus in Swine Is Mediated by IL-10. *Plos One* 2009;4:11.

223. Parkin J, Cohen B. An overview of the immune system. *Lancet* 2001;357:1777-1789.

224. Gauduin MC, Glickman RL, Ahmad S, et al. Characterization of SIV-specific CD4(+) T-helper proliferative responses in macaques immunized with live-attenuated SIV. *Journal of Medical Primatology* 1999;28:233-241.

225. Quade MJ, Roth JA. Dual-color flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen-stimulated bovine lymphocyte subsets. *Veterinary Immunology and Immunopathology* 1999;67:33-45.

226. Pesoa S, Martin A, Marian AL, et al. Interleukin 2 induction of proliferation in resting T lymphocytes requires contact with monocytes. *Medicina-Buenos Aires* 2000;60:202-210.

227. Fukao T, Koyasu S. Expression of functional IL-2 receptors on mature splenic dendritic cells. *European Journal of Immunology* 2000;30:1453-1457.

228. Endsley JJ, Quade MJ, Terhaar B, et al. BHV-1-specific CD4(+), CD8(+), and gamma delta T cells in calves vaccinated with one dose of a modified live BHV-1 vaccine. *Viral Immunology* 2002;15:385-393.

229. Ohmura M, Yamamoto M, Tomiyama-Miyaji C, et al. Nontoxic Shiga toxin derivatives from *Escherichia coli* possess adjuvant activity for the augmentation of antigen-specific immune responses via dendritic cell activation. *Infection and Immunity* 2005;73:4088-4097.

230. Haicheur N, Benchetrit F, Amessou M, et al. The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a T(h)1-dominant polarization. *International Immunology* 2003;15:1161-1171.

231. Pellino CA, Karve SS, Pradhan S, et al. AB(5) Preassembly Is Not Required for Shiga Toxin Activity. *Journal of Bacteriology* 2016;198:1621-1630.

232. Le Nours J, Paton AW, Byres E, et al. Structural Basis of Subtilase Cytotoxin SubAB Assembly. *Journal of Biological Chemistry* 2013;288:27505-27516.

233. Donohuerolfe A, Jacewicz M, Keusch GT. Isolation and characterization of functional shiga toxin subunits and renatured holotoxin. *Molecular Microbiology* 1989;3:1231-1236.

234. Meyer G, Deplanche M, Schelcher F. Human and bovine respiratory syncytial virus vaccine research and development. *Comparative Immunology Microbiology and Infectious Diseases* 2008;31:191-225.

235. Mahajan A, Currie CG, Mackie S, et al. An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157: H7 with bovine intestinal epithelium. *Cellular Microbiology* 2009;11:121-137.

236. Naylor SW, Flockhart A, Nart P, et al. Shedding of *Escherichia coli* O157 : H7 in calves is reduced by prior colonization with the homologous strain. *Applied and Environmental Microbiology* 2007;73:3765-3767.

237. Sanderson MW, Besser TE, Gay JM, et al. Fecal *Escherichia coli* O157 : H7 shedding patterns of orally inoculated calves. *Veterinary Microbiology* 1999;69:199-205.

238. Arthur TM, Ahmed R, Chase-Topping M, et al. Characterization of *Escherichia coli* O157:H7 strains isolated from supershedding cattle. *Appl Environ Microbiol* 2013;79:4294-4303.

239. Williams KJ, Ward MP, Dhungyel OP, et al. Risk factors for *Escherichia coli* O157 shedding and super-shedding by dairy heifers at pasture. *Epidemiology and Infection* 2015;143:1004-1015.

240. Xu Y, Dugat-Bony E, Zaheer R, et al. *Escherichia coli* O157:H7 super-shedder and non-shedder feedlot steers harbour distinct fecal bacterial communities. *PLoS One* 2014;9:e98115.

241. Zaheer R, Dugat-Bony E, Holman DB, et al. Changes in bacterial community composition of *Escherichia coli* O157:H7 super-shedder cattle occur in the lower intestine. *PLoS One* 2017;12:e0170050.

242. Tedder TE, Engel P. CD20 - A REGULATOR OF CELL-CYCLE PROGRESSION OF B-LYMPHOCYTES. *Immunology Today* 1994;15:450-454.

243. Kuijpers TW, Bende RJ, Baars PA, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. *Journal of Clinical Investigation* 2010;120:214-222.

244. Nagira M, Imai T, Yoshida R, et al. A lymphocyte-specific CC chemokine, secondary lymphoid tissue chemokine (SLC), is a highly efficient chemoattractant for B cells and activated T cells. *European Journal of Immunology* 1998;28:1516-1523.

245. Riol-Blanco L, Sanchez-Sanchez N, Torres A, et al. The chemokine receptor CCR7 activates in dendritic cells two signaling modules that independently regulate chemotaxis and migratory speed. *Journal of Immunology* 2005;174:4070-4080.

246. Nagy N, Cerboni C, Mattsson K, et al. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. *Int J Cancer* 2000;88:439-447.

247. Poe JC, Fujimoto Y, Hasegawa M, et al. CD22 regulates B lymphocyte function *in vivo* through both ligand-dependent and ligand-independent mechanisms. *Nature Immunology* 2004;5:1078-1087.

248. Sato S, Tuscano JM, Inaoki M, et al. CD22 negatively and positively regulates signal transduction through the B lymphocyte antigen receptor. *Seminars in Immunology* 1998;10:287-297.

249. Weiss A, Imboden JB. Cell-surface molecules and early events involved in human lymphocyte-T activation. *Advances in Immunology* 1987;41:1-38.

250. Wakasugi H, Bertoglio J, Tursz T, et al. IL-2 receptor induction on human lymphocytes-T - role for IL-2 and monocytes. *Journal of Immunology* 1985;135:321-327.

251. Robertson MJ, Manley TJ, Donahue C, et al. Costimulatory signals are required for optimal proliferation of human natural-killer cells. *Journal of Immunology* 1993;150:1705-1714.

252. Burchill MA, Yang J, Vang KB, et al. Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. *Immunol Lett* 2007;114:1-8.

253. Browning JL, Ngamek A, Lawton P, et al. Lymphotoxin-beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 1993;72:847-856.

254. Crowe PD, Vanarsdale TL, Walter BN, et al. A lymphotoxin -beta specific receptor. *Science* 1994;264:707-710.

255. McDonald KG, McDonough JS, Newberry RD. Adaptive immune responses are dispensable for isolated lymphoid follicle formation: Antigen-naive, lymphotoxin-sufficient B lymphocytes drive the formation of mature isolated lymphoid follicles. *Journal of Immunology* 2005;174:5720-5728.

256. Forster R, Schubel A, Breitfeld D, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999;99:23-33.

257. Sallusto F, Schaerli P, Loetscher P, et al. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *European Journal of Immunology* 1998;28:2760-2769.

258. Haas KM, Tedder TF. Role of the CD19 and CD21/35 receptor complex in innate immunity, host defense and autommunity In: Gupta S, Paul WE,Steinman R, eds. *Mechanisms of Lymphocyte Activation and Immune Regulation X: Innate Immunity*. New York: Springer, 2005;125-139.

259. Karnell JL, Dimasi N, Karnell FG, et al. CD19 and CD32b Differentially Regulate Human B Cell Responsiveness. *Journal of Immunology* 2014;192:1480-1490.

260. Nielsen PJ, Georgiev O, Lorenz B, et al. B lymphocytes are impaired in mice lacking the transcriptional co-activator Bob1/OCA-B/OBF1. *European Journal of Immunology* 1996;26:3214-3218.

261. Bowman EP, Campbell JJ, Soler D, et al. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *Journal of Experimental Medicine* 2000;191:1303-1317.

262. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000;406:309-314.

263. Ansel KM, Harris RBS, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* 2002;16:67-76.

264. St John AL, Abraham SN. Salmonella disrupts lymph node architecture by TLR4-mediated suppression of homeostatic chemokines. *Nature Medicine* 2009;15:1259-U1251.

265. Fries PN, Popowych YI, Guan LL, et al. Age-related changes in the distribution and frequency of myeloid and T cell populations in the small intestine of calves. *Cell Immunol* 2011;271:428-437.

266. Liang G, Malmuthuge N, Bao H, et al. Transcriptome analysis reveals regional and temporal differences in mucosal immune system development in the small intestine of neonatal calves. *BMC Genomics* 2016;17:602.

267. Malmuthuge N, Li M, Goonewardene LA, et al. Effect of calf starter feeding on gut microbial diversity and expression of genes involved in host immune responses and tight junctions in dairy calves during weaning transition. *J Dairy Sci* 2013;96:3189-3200.

268. Uchida J, Lee Y, Hasegawa M, et al. Mouse CD20 expression and function. *International Immunology* 2004;16:119-129.

269. Dong ZJ, Davidson D, Perez-Quintero LA, et al. The Adaptor SAP Controls NK Cell Activation by Regulating the Enzymes Vav-1 and SHIP-1 and by Enhancing Conjugates with Target Cells. *Immunity* 2012;36:974-985.

270. Jang MH, Sougawa N, Tanaka T, et al. CCR7 is critically important in intestinal lamina propria for migration of dendritic cells to mesenteric lymph nodes. *Journal of Immunology* 2006;176:803-810.

271. Li J, Hovde CJ. Expression profiles of bovine genes in the rectoanal junction mucosa during colonization with *Escherichia coli* O157 : H7. *Applied and Environmental Microbiology* 2007;73:2380-2385.

272. Carreno BM, Collins M. The B7 family of ligands and its receptors: New pathways for costimulation and inhibition of immune responses. *Annual Review of Immunology* 2002;20:29-53.

273. Azuma M, Ito D, Yagita H, et al. B70 antigen is a 2nd ligand for CTLA-4 and CD28. *Nature* 1993;366:76-79.

274. Lanier LL, Ofallon S, Somoza C, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T-cell proliferation, cytokine production, and generation of CTL. *Journal of Immunology* 1995;154:97-105.

275. Caux C, Vanbervliet B, Massacrier C, et al. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *Journal of Experimental Medicine* 1994;180:1841-1847.

276. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:Research0034.

277. Keen JE, Laegreid WW, Chitko-McKown CG, et al. Distribution of Shiga-Toxigenic *Escherichia coli* O157 in the Gastrointestinal Tract of Naturally O157-Shedding Cattle at Necropsy. *Applied and Environmental Microbiology* 2010;76:5278-5281.

278. Huggett J, Dheda K, Bustin S, et al. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity* 2005;6:279-284.

279. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 2001;25:402-408.

280. Wang O, McAllister TA, Plastow G, et al. Host mechanisms involved in cattle *Escherichia coli* O157 shedding: a fundamental understanding for reducing foodborne pathogen in food animal production. *Scientific Reports* 2017;7:15.

281. Baines D, Lee B, McAllister T. Heterogeneity in enterohemorrhagic *Escherichia coli* O157:H7 fecal shedding in cattle is related to *Escherichia coli* O157:H7 colonization of the small and large intestine. *Canadian Journal of Microbiology* 2008;54:984-995.

282. Lammers GAC, Jordan D, McConnel CS, et al. Daily shedding dynamics of *E-coli* O157 in an Australian grass-fed beef herd. *Epidemiology and Infection* 2016;144:2948-2955.

283. Munns KD, Selinger L, Stanford K, et al. Are Super-Shedder Feedlot Cattle Really Super? *Foodborne Pathogens and Disease* 2014;11:329-331.

284. Besser TE, Hancock DD, Pritchett LC, et al. Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *Journal of Infectious Diseases* 1997;175:726-729.

285. Sanderson MW, Sargeant JM, Shi XR, et al. Longitudinal emergence and distribution of *Escherichia coli* O157 genotypes in a beef feedlot. *Applied and Environmental Microbiology* 2006;72:7614-7619.

286. Renter DG, Sargeant JM, Oberst RD, et al. Diversity, frequency, and persistence of *Escherichia coli* O157 strains from range cattle environments. *Applied and Environmental Microbiology* 2003;69:542-547.

287. Olejniczak K, Kasprzak A. Biological properties of interleukin 2 and its role in pathogenesis of selected diseases - a review. *Medical Science Monitor* 2008;14:RA179-RA189.

288. Nelson BH. IL-2, regulatory T cells, and tolerance. *Journal of Immunology* 2004;172:3983-3988.

289. Massot M, Couffignal C, Clermont O, et al. Day-to-Day Dynamics of Commensal *Escherichia coli* in Zimbabwean Cows Evidence Temporal Fluctuations within a Host-Specific Population Structure. *Applied and Environmental Microbiology* 2017;83:14.

290. McNeilly TN, Mitchell MC, Rosser T, et al. Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of *Escherichia coli* O157:H7 following oral challenge. *Vaccine* 2010;28:1422-1428.

291. Mulcahy G, Reid E, Dimarchi RD, et al. MATURATION OF FUNCTIONAL ANTIBODY-AFFINITY IN ANIMALS IMMUNIZED WITH SYNTHETIC FOOT-AND-MOUTH-DISEASE VIRUS. *Research in Veterinary Science* 1992;52:133-140.

292. Kenney JS, Hughes BW, Masada MP, et al. INFLUENCE OF ADJUVANTS ON THE QUANTITY, AFFINITY, ISOTYPE AND EPITOPE SPECIFICITY OF MURINE ANTIBODIES. *Journal of Immunological Methods* 1989;121:157-166.

293. LeJeune JT, Hancock DD, Besser TE. Sensitivity of *Escherichia coli* O157 detection in bovine feces assessed by broth enrichment followed by immunomagnetic separation and direct plating methodologies. *Journal of Clinical Microbiology* 2006;44:872-875.

294. Carlson BA, Nightingale KK, Mason GL, et al. *Escherichia coli* O157:H7 Strains That Persist in Feedlot Cattle Are Genetically Related and Demonstrate an Enhanced Ability To Adhere to Intestinal Epithelial Cells. *Applied and Environmental Microbiology* 2009;75:5927-5937.

295. Park D, Stanton E, Ciezki K, et al. Evolution of the Stx2-Encoding Prophage in Persistent Bovine *Escherichia coli* O157:H7 Strains. *Applied and Environmental Microbiology* 2013;79:1563-1572.

Appendix

Table 15: Test results for the fixed effects of the linear mixed models used to analyse changes in gene expression at the terminal rectum for calves challenged with PT21/28 Stx2c+ or unchallenged controls (treatment groups).

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CCL21	Log_{10} (Expression pre-challenge)	1	6	5.387	0.053
CCL21	Treatment	1	6	0.390	0.552
CCL21	Day Post Challenge (DPC)	1	7	0.096	0.766
CCL21	Treatment*DPC	1	7	0.676	0.435
CCR7	Log_{10} (Expression pre-challenge)	1	6	11.215	0.012
CCR7	Treatment	1	6	1.902	0.210
CCR7	Day Post Challenge (DPC)	1	7	0.014	0.910
CCR7	Treatment*DPC	1	7	0.807	0.395
CD19	Log_{10} (Expression pre-challenge)	1	6	5.860	0.046
CD19	Treatment	1	6	0.555	0.481
CD19	Day Post Challenge (DPC)	1	7	0.007	0.937
CD19	Treatment*DPC	1	7	1.774	0.220
CD22	Log_{10} (Expression pre-challenge)	1	6	4.876	0.063
CD22	Treatment	1	6	0.005	0.946
CD22	Day Post Challenge (DPC)	1	7	0.045	0.838
CD22	Treatment*DPC	1	7	3.401	0.108
CD80	Log_{10} (Expression pre-challenge)	1	6	26.810	0.001
CD80	Treatment	1	6	1.104	0.328

Table 15 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CD80	Day Post Challenge (DPC)	1	7	38.206	0.000
CD80	Treatment*DPC	1	7	0.011	0.920
CD86	Log_{10} (Expression pre-challenge)	1	6	7.883	0.026
CD86	Treatment	1	6	0.552	0.482
CD86	Day Post Challenge (DPC)	1	7	5.253	0.051
CD86	Treatment*DPC	1	7	0.766	0.407
CXCL13	Log_{10} (Expression pre-challenge)	1	6	5.343	0.054
CXCL13	Treatment	1	6	2.176	0.184
CXCL13	Day Post Challenge (DPC)	1	7	0.122	0.736
CXCL13	Treatment*DPC	1	7	2.840	0.130
IL2RA	Log_{10} (Expression pre-challenge)	1	6	9.768	0.017
IL2RA	Treatment	1	6	2.004	0.200
IL2RA	Day Post Challenge (DPC)	1	7	2.274	0.170
IL2RA	Treatment*DPC	1	7	3.430	0.101
LTB	Log_{10} (Expression pre-challenge)	1	6	36.405	0.001
LTB	Treatment	1	6	1.258	0.299
LTB	Day Post Challenge (DPC)	1	7	2.716	0.138
LTB	Treatment*DPC	1	7	0.464	0.515
MS4A1	Log_{10} (Expression pre-challenge)	1	6	5.928	0.045
MS4A1	Treatment	1	6	2.772	0.140

Table 15 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
MS4A1	Day Post Challenge (DPC)	1	7	0.670	0.437
MS4A1	Treatment*DPC	1	7	1.502	0.255
POU2AF1	Log_{10} (Expression pre-challenge)	1	6	7.784	0.027
POU2AF1	Treatment	1	6	0.770	0.409
POU2AF1	Day Post Challenge (DPC)	1	7	0.425	0.533
POU2AF1	Treatment*DPC	1	7	1.650	0.235
SH2D1A	Log_{10} (Expression pre-challenge)	1	6	5.550	0.051
SH2D1A	Treatment	1	6	0.301	0.600
SH2D1A	Day Post Challenge (DPC)	1	7	0.119	0.739
SH2D1A	Treatment*DPC	1	7	2.982	0.123

Table 16: Test results for the fixed effects of the linear mixed models used to analyse changes in gene expression at the terminal rectum for calves challenged with PT32 Stx2c+ or unchallenged controls (treatment groups).

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CCL21	Log_{10} (Expression pre-challenge)	1	6	1.847	0.223
CCL21	Treatment	1	6	0.506	0.504
CCL21	Day Post Challenge (DPC)	1	7	3.3878	0.090
CCL21	Treatment*DPC	1	7	0.599	0.464
CCR7	Log_{10} (Expression pre-challenge)	1	6	5.270	0.062
CCR7	Treatment	1	6	0.191	0.678
CCR7	Day Post Challenge (DPC)	1	7	2.891	0.133

Table 16 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CCR7	Treatment*DPC	1	7	0.084	0.780
CD19	Log_{10} (Expression pre-challenge)	1	6	0.025	0.880
CD19	Treatment	1	6	0.004	0.950
CD19	Day Post Challenge (DPC)	1	7	3.653	0.098
CD19	Treatment*DPC	1	7	0.243	0.637
CD22	Log_{10} (Expression pre-challenge)	1	6	1.207	0.314
CD22	Treatment	1	6	1.480	0.270
CD22	Day Post Challenge (DPC)	1	7	2.792	0.139
CD22	Treatment*DPC	1	7	0.032	0.863
CD80	Log_{10} (Expression pre-challenge)	1	6	0.681	0.441
CD80	Treatment	1	6	2.549	0.162
CD80	Day Post Challenge (DPC)	1	7	17.524	0.004
CD80	Treatment*DPC	1	7	0.056	0.820
CD86	Log_{10} (Expression pre-challenge)	1	6	0.383	0.559
CD86	Treatment	1	6	0.080	0.786
CD86	Day Post Challenge (DPC)	1	7	32.601	0.001
CD86	Treatment*DPC	1	7	0.299	0.601
CXCL13	Log_{10} (Expression pre-challenge)	1	6	2.747	0.149
CXCL13	Treatment	1	6	0.327	0.588
CXCL13	Day Post Challenge (DPC)	1	7	0.407	0.544

Table 16 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*- value
CXCL13	Treatment*DPC	1	7	0.624	0.456
IL2RA	Log_{10} (Expression pre-challenge)	1	6	2.754	0.148
IL2RA	Treatment	1	6	3.282	0.120
IL2RA	Day Post Challenge (DPC)	1	7	9.570	0.018
IL2RA	Treatment*DPC	1	7	1.006	0.349
LTB	Log_{10} (Expression pre-challenge)	1	6	0.257	0.603
LTB	Treatment	1	6	0.301	0.603
LTB	Day Post Challenge (DPC)	1	7	11.961	0.011
LTB	Treatment*DPC	1	7	0.014	0.911
MS4A1	Log_{10} (Expression pre-challenge)	1	6	4.467	0.079
MS4A1	Treatment	1	6	0.608	0.465
MS4A1	Day Post Challenge (DPC)	1	7	7.109	0.032
MS4A1	Treatment*DPC	1	7	0.229	0.647
POU2AF1	Log_{10} (Expression pre-challenge)	1	6	1.772	0.232
POU2AF1	Treatment	1	6	0.201	0.669
POU2AF1	Day Post Challenge (DPC)	1	7	2.182	0.183
POU2AF1	Treatment*DPC	1	7	0.146	0.714
SH2D1A	Log_{10} (Expression pre-challenge)	1	6	1.909	0.216
SH2D1A	Treatment	1	6	0.009	0.928
SH2D1A	Day Post Challenge (DPC)	1	7	2.919	0.131

Table 16 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
SH2D1A	Treatment*DPC	1	7	0.285	0.610

Table 17: Test results for the fixed effects of the linear mixed models used to analyse changes in gene expression at the terminal rectum for calves challenged with PT21/28 Stx2a+Stx2c+ or unchallenged controls (treatment groups).

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CCL21	Log_{10} (Expression pre-challenge)	1	6	12.222	0.007
CCL21	Treatment	1	6	0.062	0.809
CCL21	Day Post Challenge (DPC)	1	7	1.954	0.192
CCL21	Treatment*DPC	1	7	0.003	0.957
CCR7	Log_{10} (Expression pre-challenge)	1	6	18.033	0.002
CCR7	Treatment	1	6	0.036	0.854
CCR7	Day Post Challenge (DPC)	1	7	1.808	0.209
CCR7	Treatment*DPC	1	7	2.535	0.142
CD19	Log_{10} (Expression pre-challenge)	1	6	36.219	0.000
CD19	Treatment	1	6	0.121	0.736
CD19	Day Post Challenge (DPC)	1	7	1.304	0.280
CD19	Treatment*DPC	1	7	3.324	0.098
CD22	Log_{10} (Expression pre-challenge)	1	6	29.850	0.000
CD22	Treatment	1	6	0.309	0.592
CD22	Day Post Challenge (DPC)	1	7	0.104	0.754
CD22	Treatment*DPC	1	7	3.604	0.087

Table 17 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
CD80	Log_{10} (Expression pre-challenge)	1	6	37.862	0.000
CD80	Treatment	1	6	0.118	0.739
CD80	Day Post Challenge (DPC)	1	7	59.697	0.000
CD80	Treatment*DPC	1	7	1.917	0.196
CD86	Log_{10} (Expression pre-challenge)	1	6	57.167	0.000
CD86	Treatment	1	6	0.056	0.819
CD86	Day Post Challenge (DPC)	1	7	11.161	0.008
CD86	Treatment*DPC	1	7	2.753	0.128
CXCL13	Log_{10} (Expression pre-challenge)	1	6	19.239	0.002
CXCL13	Treatment	1	6	0.90	0.771
CXCL13	Day Post Challenge (DPC)	1	7	0.983	0.345
CXCL13	Treatment*DPC	1	7	2.958	0.116
IL2RA	Log_{10} (Expression pre-challenge)	1	6	11.549	0.008
IL2RA	Treatment	1	6	0.191	0.673
IL2RA	Day Post Challenge (DPC)	1	7	4.254	0.066
IL2RA	Treatment*DPC	1	7	7.733	0.019
LTB	Log_{10} (Expression pre-challenge)	1	6	32.064	0.000
LTB	Treatment	1	6	0.306	0.594
LTB	Day Post Challenge (DPC)	1	7	6.810	0.026
LTB	Treatment*DPC	1	7	1.432	0.259

Table 17 continued

Gene	Fixed Effect	Number DF	Den DF	F-value	*p*-value
MS4A1	Log_{10} (Expression pre-challenge)	1	6	16.010	0.003
MS4A1	Treatment	1	6	0.014	0.907
MS4A1	Day Post Challenge (DPC)	1	7	0.000	0.986
MS4A1	Treatment*DPC	1	7	1.236	0.292
POU2AF1	Log_{10} (Expression pre-challenge)	1	6	32.251	0.000
POU2AF1	Treatment	1	6	2.451	0.152
POU2AF1	Day Post Challenge (DPC)	1	7	1.317	0.278
POU2AF1	Treatment*DPC	1	7	0.002	0.936
SH2D1A	Log_{10} (Expression pre-challenge)	1	6	43.512	0.000
SH2D1A	Treatment	1	6	0.951	0.355
SH2D1A	Day Post Challenge (DPC)	1	7	0.421	0.531
SH2D1A	Treatment*DPC	1	7	0.360	0.562

Table 18: Predicted group means (fold change in log_{10} scale) and 95 % Confidence intervals (CIs) from the linear mixed models used to analyse changes in gene expression at the terminal rectum.

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
CCL21	PT21/28 Stx2c+ challenged	7	-0.302	-1.185	0.582
CCL21	PT21/28 Stx2c+ controls	7	-0.383	-1.516	0.751
CCL21	PT32 Stx2c+ challenged	7	1.352	0.635	2.068
CCL21	PT32 Stx2c+ controls	7	1.372	0.337	2.406
CCL21	PT21/28 Stx2a+Stx2c+ challenged	7	0.213	-0.725	1.151
CCL21	PT21/28 Stx2a+Stx2c+ controls	7	0.093	-0.896	1.081
CCL21	PT21/28 Stx2c+ challenged	26	-0.745	-1.629	0.139
CCL21	PT21/28 Stx2c+ controls	26	-0.068	-1.201	1.066
CCL21	PT32 Stx2c+ challenged	26	0.808	0.092	1.525
CCL21	PT32 Stx2c+ controls	26	0.201	-0.833	1.235
CCL21	PT21/28 Stx2a+Stx2c+ challenged	26	-0.248	-1.186	0.690
CCL21	PT21/28 Stx2a+Stx2c+ controls	26	-0.407	-1.395	0.582
CCR7	PT21/28 Stx2c+ challenged	7	-0.170	-0.958	0.619
CCR7	PT21/28 Stx2c+ controls	7	0.049	-0.964	1.063
CCR7	PT32 Stx2c+ challenged	7	1.120	0.416	1.983
CCR7	PT32 Stx2c+ controls	7	1.542	0.358	2.725
CCR7	PT21/28 Stx2a+Stx2c+ challenged	7	0.264	-0.491	1.018

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
CCR7	PT21/28 Stx2a+Stx2c+ controls	7	0.877	0.053	1.702
CCR7	PT21/28 Stx2c+ challenged	26	-0.419	-1.207	0.370
CCR7	PT21/28 Stx2c+ controls	26	0.540	-0.474	1.554
CCR7	PT32 Stx2c+ challenged	26	0.574	-0.210	1.357
CCR7	PT32 Stx2c+ controls	26	0.658	-0.525	1.842
CCR7	PT21/28 Stx2a+Stx2c+ challenged	26	0.264	-0.490	1.018
CCR7	PT21/28 Stx2a+Stx2c+ controls	26	-0.191	-1.015	0.634
CD19	PT21/28 Stx2c+ challenged	7	-0.442	-1.202	0.319
CD19	PT21/28 Stx2c+ controls	7	-0.670	-1.610	0.269
CD19	PT32 Stx2c+ challenged	7	0.923	0.282	1.563
CD19	PT32 Stx2c+ controls	7	1.126	0.197	2.055
CD19	PT21/28 Stx2a+Stx2c+ challenged	7	0.023	-0.605	0.651
CD19	PT21/28 Stx2a+Stx2c+ controls	7	0.708	-0.002	1.418
CD19	PT21/28 Stx2c+ challenged	26	-0.828	-1.588	-0.068
CD19	PT21/28 Stx2c+ controls	26	-0.013	-0.952	0.926
CD19	PT32 Stx2c+ challenged	26	0.388	-0.253	1.029
CD19	PT32 Stx2c+ controls	26	0.233	-0.695	1.162

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
CD19	PT21/28 Stx2a+Stx2c+ challenged	26	0.146	-0.482	0.774
CD19	PT21/28 Stx2a+Stx2c+ controls	26	-0.312	-1.022	0.397
CD22	PT21/28 Stx2c+ challenged	7	-0.242	-1.097	0.613
CD22	PT21/28 Stx2c+ controls	7	-0.962	-2.000	0.076
CD22	PT32 Stx2c+ challenged	7	1.053	0.182	1.923
CD22	PT32 Stx2c+ controls	7	1.765	0.502	3.028
CD22	PT21/28 Stx2a+Stx2c+ challenged	7	0.051	-0.738	0.841
CD22	PT21/28 Stx2a+Stx2c+ controls	7	1.025	0.130	1.920
CD22	PT21/28 Stx2c+ challenged	26	-0.753	-1.608	0.101
CD22	PT21/28 Stx2c+ controls	26	0.250	-1.038	1.539
CD22	PT32 Stx2c+ challenged	26	0.346	-0.524	1.217
CD22	PT32 Stx2c+ controls	26	0.886	-0.377	2.150
CD22	PT21/28 Stx2a+Stx2c+ challenged	26	0.550	-0.239	1.340
CD22	PT21/28 Stx2a+Stx2c+ controls	26	0.025	-0.870	0.920
CD80	PT21/28 Stx2c+ challenged	7	0.436	0.052	0.819
CD80	PT21/28 Stx2c+ controls	7	0.191	-0.255	0.636

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
CD80	PT32 Stx2c+ challenged	7	0.591	0.098	1.084
CD80	PT32 Stx2c+ controls	7	0.150	-0.538	0.837
CD80	PT21/28 Stx2a+Stx2c+ challenged	7	0.451	0.209	0.694
CD80	PT21/28 Stx2a+Stx2c+ controls	7	0.653	0.381	0.924
CD80	PT21/28 Stx2c+ challenged	26	-0.714	-1.098	-0.331
CD80	PT21/28 Stx2c+ controls	26	-0.921	-1.366	-0.475
CD80	PT32 Stx2c+ challenged	26	-0.302	-0.795	0.191
CD80	PT32 Stx2c+ controls	26	-0.855	-1.543	-0.167
CD80	PT21/28 Stx2a+Stx2c+ challenged	26	-0.259	-0.502	-0.016
CD80	PT21/28 Stx2a+Stx2c+ controls	26	-0.362	-0.633	-0.091
CD86	PT21/28 Stx2c+ challenged	7	0.124	-0.212	0.460
CD86	PT21/28 Stx2c+ controls	7	-0.157	-0.561	0.246
CD86	PT32 Stx2c+ challenged	7	0.667	0.332	1.002
CD86	PT32 Stx2c+ controls	7	0.714	0.245	1.183
CD86	PT21/28 Stx2a+Stx2c+ challenged	7	0.343	0.066	0.619
CD86	PT21/28 Stx2a+Stx2c+ controls	7	0.540	0.223	0.856
CD86	PT21/28 Stx2c+ challenged	26	-0.384	-0.721	-0.049

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
CD86	PT21/28 Stx2c+ controls	26	-0.364	-0.767	0.040
CD86	PT32 Stx2c+ challenged	26	-0.271	-0.606	0.064
CD86	PT32 Stx2c+ controls	26	-0.428	-0.897	0.041
CD86	PT21/28 Stx2a+Stx2c+ challenged	26	0.076	-0.201	0.352
CD86	PT21/28 Stx2a+Stx2c+ controls	26	-0.191	-0.507	0.126
CXCL13	PT21/28 Stx2c+ challenged	7	-0.414	-1.287	0.459
CXCL13	PT21/28 Stx2c+ controls	7	-0.466	-1.605	0.673
CXCL13	PT32 Stx2c+ challenged	7	0.881	0.210	1.553
CXCL13	PT32 Stx2c+ controls	7	0.963	0.012	1.916
CXCL13	PT21/28 Stx2a+Stx2c+ challenged	7	0.309	-0.483	1.101
CXCL13	PT21/28 Stx2a+Stx2c+ controls	7	1.009	0.133	1.878
CXCL13	PT21/28 Stx2c+ challenged	26	-1.188	-2.061	-0.315
CXCL13	PT21/28 Stx2c+ controls	26	0.303	-0.836	1.442
CXCL13	PT32 Stx2c+ challenged	26	0.853	0.181	1.525
CXCL13	PT32 Stx2c+ controls	26	0.335	-0.617	1.287
CXCL13	PT21/28 Stx2a+Stx2c+ challenged	26	0.458	-0.335	1.250
CXCL13	PT21/28 Stx2a+Stx2c+ controls	26	0.034	-0.835	0.904

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
IL2RA	PT21/28 Stx2c+ challenged	7	-0.795	-1.522	-0.067
IL2RA	PT21/28 Stx2c+ controls	7	-0.910	-1.878	0.058
IL2RA	PT32 Stx2c+ challenged	7	0.441	-0.739	1.620
IL2RA	PT32 Stx2c+ controls	7	0.982	-0.689	2.654
IL2RA	PT21/28 Stx2a+Stx2c+ challenged	7	-1.793	-2.987	-0.600
IL2RA	PT21/28 Stx2a+Stx2c+ controls	7	0.056	-1.480	1.591
IL2RA	PT21/28 Stx2c+ challenged	26	-0.796	-1.524	-0.069
IL2RA	PT21/28 Stx2c+ controls	26	0.501	-0.467	1.469
IL2RA	PT32 Stx2c+ challenged	26	-1.953	-3.132	-0.773
IL2RA	PT32 Stx2c+ controls	26	-0.072	-1.744	1.600
IL2RA	PT21/28 Stx2a+Stx2c+ challenged	26	0.602	-0.591	1.795
IL2RA	PT21/28 Stx2a+Stx2c+ controls	26	-0.611	-2.146	0.925
LTB	PT21/28 Stx2c+ challenged	7	-0.342	-0.634	-0.050
LTB	PT21/28 Stx2c+ controls	7	-0.271	-0.640	0.097
LTB	PT32 Stx2c+ challenged	7	0.863	0.312	1.413
LTB	PT32 Stx2c+ controls	7	0.655	-0.125	1.435
LTB	PT21/28 Stx2a+Stx2c+ challenged	7	0.127	-0.206	0.460

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
LTB	PT21/28 Stx2a+Stx2c+ controls	7	0.425	-0.043	0.808
LTB	PT21/28 Stx2c+ challenged	26	-0.671	-0.963	-0.379
LTB	PT21/28 Stx2c+ controls	26	-0.393	-0.762	-0.024
LTB	PT32 Stx2c+ challenged	26	-0.177	-0.727	0.373
LTB	PT32 Stx2c+ controls	26	-0.312	-1.092	0.468
LTB	PT21/28 Stx2a+Stx2c+ challenged	26	-0.140	-0.473	0.193
LTB	PT21/28 Stx2a+Stx2c+ controls	26	-0.247	-0.629	0.136
MS4A1	PT21/28 Stx2c+ challenged	7	-0.586	-1.545	0.373
MS4A1	PT21/28 Stx2c+ controls	7	-0.340	-1.566	0.887
MS4A1	PT32 Stx2c+ challenged	7	0.631	-0.029	1.291
MS4A1	PT32 Stx2c+ controls	7	0.744	-0.192	1.680
MS4A1	PT21/28 Stx2a+Stx2c+ challenged	7	0.387	-0.387	1.161
MS4A1	PT21/28 Stx2a+Stx2c+ controls	7	0.812	-0.046	1.671
MS4A1	PT21/28 Stx2c+ challenged	26	-0.676	-1.634	0.283
MS4A1	PT21/28 Stx2c+ controls	26	0.796	-0.430	2.023
MS4A1	PT32 Stx2c+ challenged	26	-0.427	-1.087	0.233
MS4A1	PT32 Stx2c+ controls	26	0.043	-0.892	0.979

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
MS4A1	PT21/28 Stx2a+Stx2c+ challenged	26	0.704	-0.070	1.478
MS4A1	PT21/28 Stx2a+Stx2c+ controls	26	0.384	-0.475	1.243
POU2AF1	PT21/28 Stx2c+ challenged	7	-0.202	-0.773	0.370
POU2AF1	PT21/28 Stx2c+ controls	7	-0.321	-1.031	0.390
POU2AF1	PT32 Stx2c+ challenged	7	0.552	-0.121	1.225
POU2AF1	PT32 Stx2c+ controls	7	0.578	-0.376	1.532
POU2AF1	PT21/28 Stx2a+Stx2c+ challenged	7	0.521	-0.380	1.422
POU2AF1	PT21/28 Stx2a+Stx2c+ controls	7	1.252	0.222	2.282
POU2AF1	PT21/28 Stx2c+ challenged	26	-0.317	-0.889	0.254
POU2AF1	PT21/28 Stx2c+ controls	26	0.328	-0.383	1.039
POU2AF1	PT32 Stx2c+ challenged	26	-0.076	-0.749	0.597
POU2AF1	PT32 Stx2c+ controls	26	0.241	0.713	1.195
POU2AF1	PT21/28 Stx2a+Stx2c+ challenged	26	1.048	0.147	1.949
POU2AF1	PT21/28 Stx2a+Stx2c+ controls	26	1.737	0.706	2.767
SH2D1A	PT21/28 Stx2c+ challenged	7	0.059	-0.483	0.601
SH2D1A	PT21/28 Stx2c+ controls	7	-0.251	-0.965	0.464
SH2D1A	PT32 Stx2c+ challenged	7	0.467	-0.184	1.118

Table 18 continued

Gene	Group	Days post challenge	Estimated fold change	95 % CIs	
				Lower	Upper
SH2D1A	PT32 Stx2c+ controls	7	0.238	-0.673	1.149
SH2D1A	PT21/28 Stx2a+Stx2c+ challenged	7	0.187	-0.529	0.903
SH2D1A	PT21/28 Stx2a+Stx2c+ controls	7	0.335	-0.458	1.129
SH2D1A	PT21/28 Stx2c+ challenged	26	-0.427	-0.967	0.115
SH2D1A	PT21/28 Stx2c+ controls	26	0.239	-0.476	0.954
SH2D1A	PT32 Stx2c+ challenged	26	-0.251	-0.902	0.400
SH2D1A	PT32 Stx2c+ controls	26	-0.091	-1.002	0.821
SH2D1A	PT21/28 Stx2a+Stx2c+ challenged	26	-0.213	-0.929	0.504
SH2D1A	PT21/28 Stx2a+Stx2c+ controls	26	0.356	-0.437	1.150

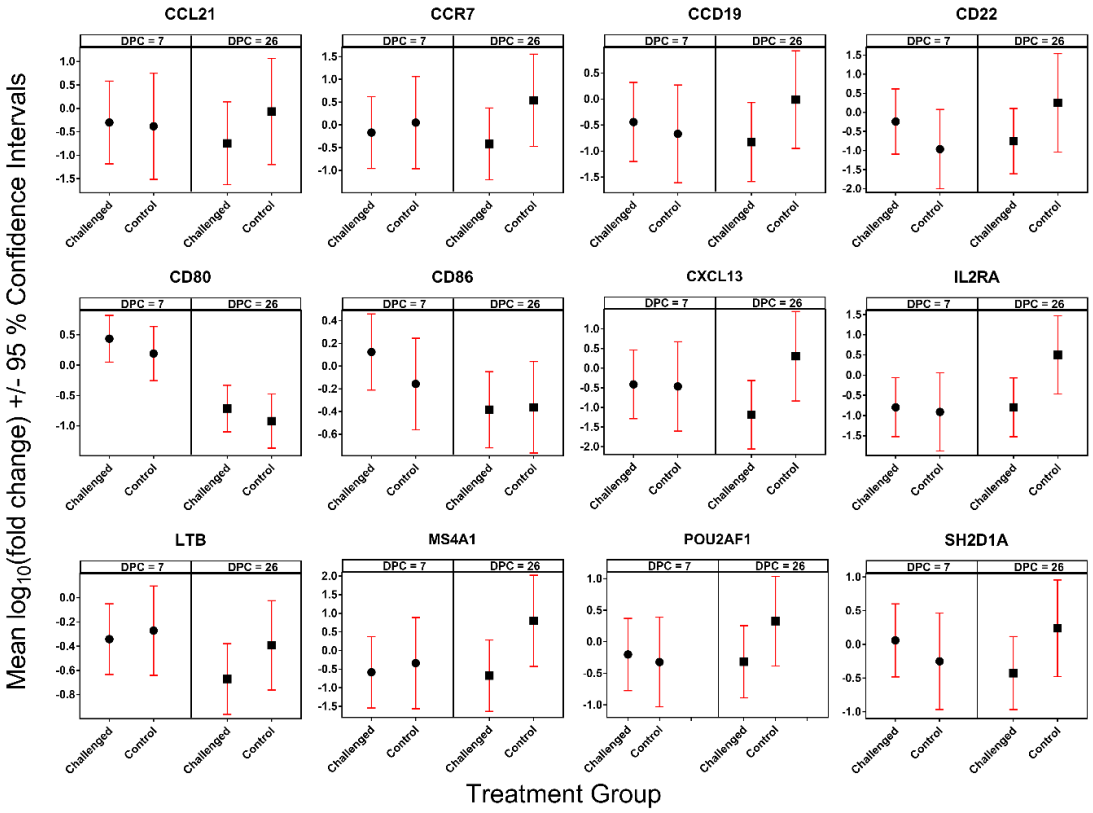


Figure 60: Predicted mean log_{10} fold change (relative to pre challenge) in transcripts for each of the 12 genes of interest, at 7 and 26 days post challenge (DPC) from the linear mixed model. Calves were orally challenged with PT21/28 Stx2c+ (challenged) or unchallenged controls (control) and rectal biopsies taken pre challenge (-3 days), 7 days and 26 days post challenge. RT-qPCR was used to determine transcript numbers for the specific immune genes, using a plasmid standard and normalisation to two housekeeping genes. Black circles represent the predicted mean log_{10} fold change (relative to pre challenged) for each group 7 days post challenge and black squares 26 days post challenge from the linear mixed model. The red error bars are 95 % confidence intervals from the linear mixed model.

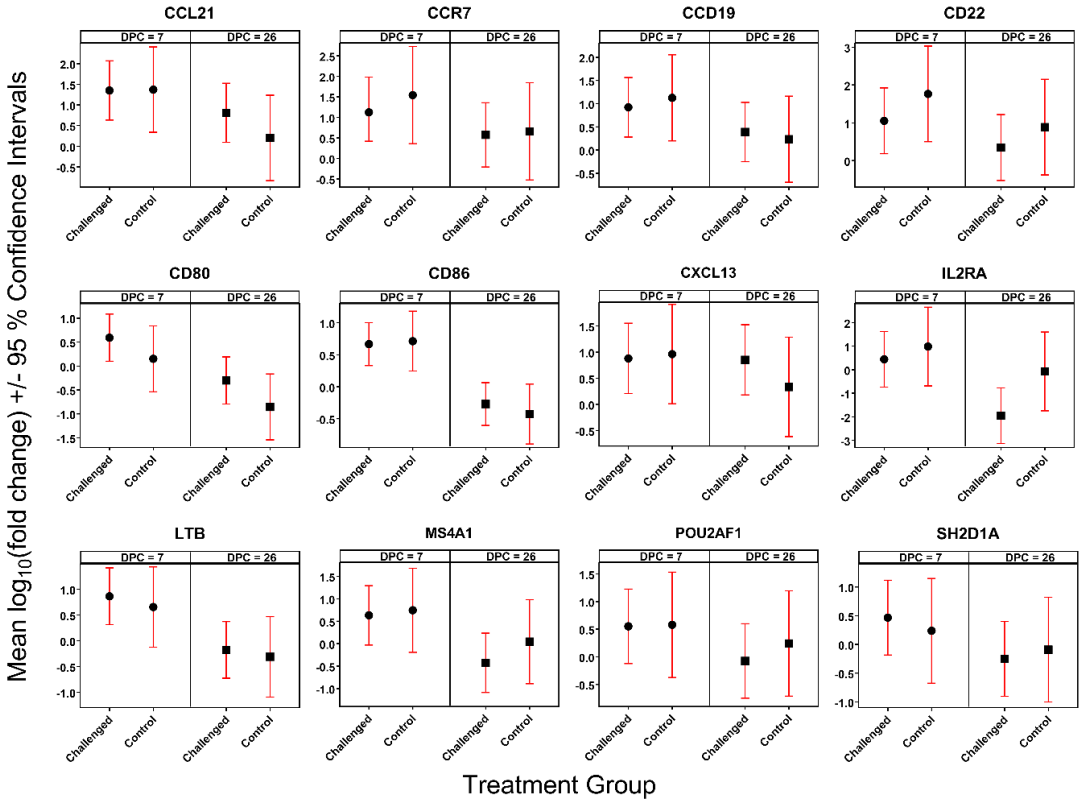


Figure 61: Predicted mean log_{10} fold change (relative to pre challenge) in transcripts for each of the 12 genes of interest, at 7 and 26 days post challenge (DPC) from the linear mixed model. Calves were orally challenged with PT32 Stx2c+ (challenged) or unchallenged controls (control) and rectal biopsies taken pre challenge (-3 days), 7 days and 26 days post challenge. RT-qPCR was used to determine transcript numbers for the specific immune genes, using a plasmid standard and normalisation to two housekeeping genes. Black circles represent the predicted mean log_{10} fold change (relative to pre challenged) for each group 7 days post challenge and black squares 26 days post challenge from the linear mixed model. The red error bars are 95 % confidence intervals from the linear mixed model.

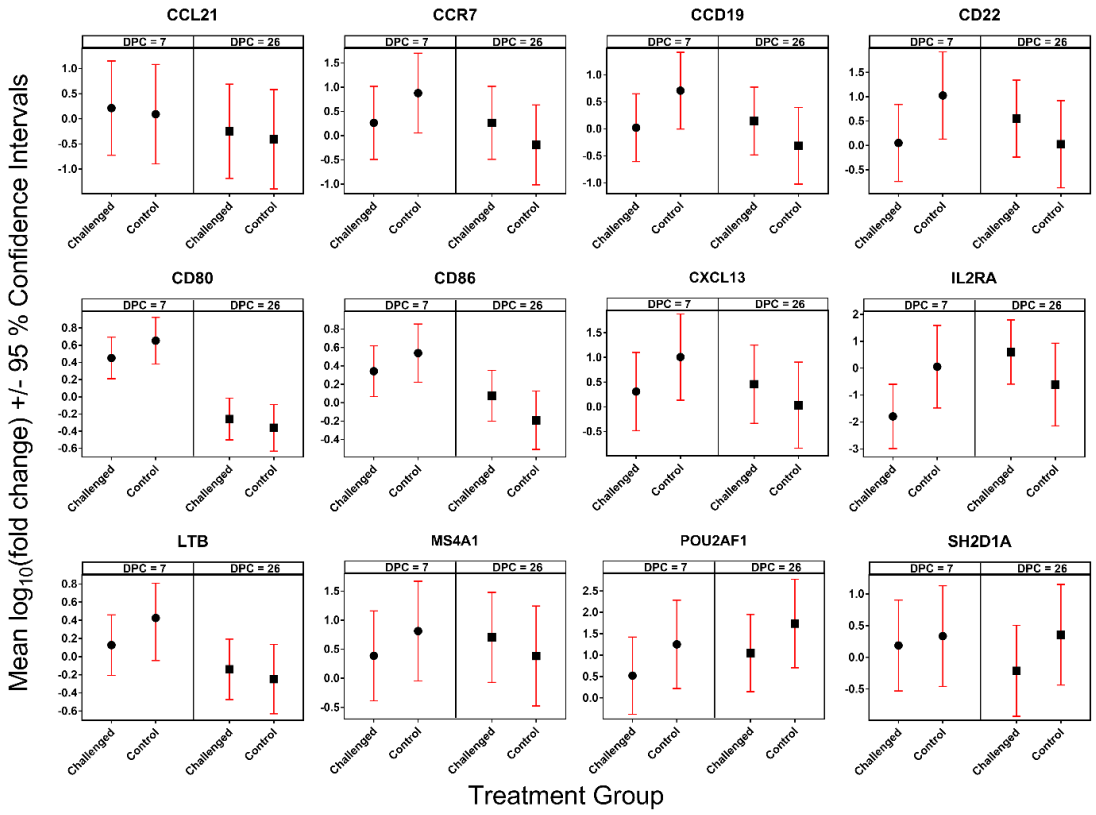


Figure 62: Predicted mean log_{10} fold change (relative to pre challenge) in transcripts for each of the 12 genes of interest, at 7 and 26 days post challenge (DPC) from the linear mixed model. Calves were orally challenged with PT21/28 Stx2a+Stx2c+ (challenged) or unchallenged controls (control) and rectal biopsies taken pre challenge (-3 days), 7 days and 26 days post challenge. RT-qPCR was used to determine transcript numbers for the specific immune genes, using a plasmid standard and normalisation to two housekeeping genes. Black circles represent the predicted mean log_{10} fold change (relative to pre challenge) for each group 7 days post challenge and black squares 26 days post challenge from the linear mixed model. The red error bars are 95 % confidence intervals from the linear mixed model.